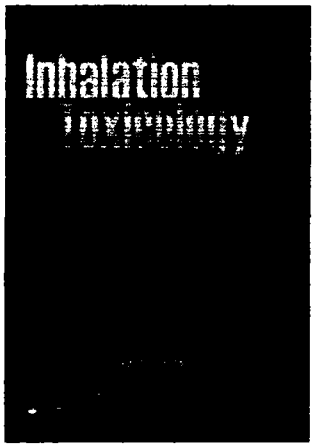


Inhalation Toxicology
International Forum for Respiratory Research
Publication details, including instructions for authors and subscription information: http://www.informaworld.com/smpp/title~content=t713657711

Testing of Fibrous Particles: Short-Term Assays and Strategies

David Bernstein; Vince Castranova [a]; Ken Donaldson [b]; Bice Fubini [c]; John Hadley [d]; Tom Hesterberg [e]; Agnes Kane [f]; David Lai [g]; Ernest E. McConnell [h]; Hartwig Muhle [i]; Gunter Oberdorster [j]; Stephen Olin [k]; David B. Warheit [l]
[a] National Institute for Occupational Safety and Health. Morgantown, West Virginia. USA
[b] University of Edinburgh Medical School. Edinburgh. UK
[c] Interdepartmental Center "G. Scansetti" for Studies on Asbestos and other Toxic Particulates, University of Torino. Italy
[d] Owens Corning Science and Technology Center. Granville, OH. USA
[e] International Truck and Engine Corp.. Warrenville, IL. USA
[f] Brown University School of Medicine. Providence, RI. USA
[g] U.S. Environmental Protection Agency. Washington, DC. USA
[h] ToxPath, Inc.. Raleigh, NC. USA
[i] Fraunhofer Institute of Toxicology and Experimental Medicine. Hannover. Germany
[j] University of Rochester School of Environmental Medicine. Rochester, NY. USA
[k] ILSI Risk Science Institute. Washington, DC. USA
[l] DuPont Haskell Laboratory for Health and Environmental Sciences. Newark, DE. USA

Online Publication Date: 01 September 2005
To link to this article: DOI: 10.1080/08958370591001121
URL: http://dx.doi.org/10.1080/08958370591001121

Inhalation Toxicology, 17:497–537, 2005

ISSN: 0895-8378 print / 1091-7691 online
DOI: 10.1080/08958370591001121

Taylor & Francis
Taylor & Francis Group

Testing of Fibrous Particles: Short-Term Assays and Strategies

Report of an ILSI Risk Science Institute Working Group

WORKING GROUP

David Bernstein (Consultant, Geneva, Switzerland)
Vince Castranova (National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA)
Ken Donaldson (University of Edinburgh Medical School, Edinburgh, UK)
Bice Fubini (Interdepartmental Center "G. Scansetti" for Studies on Asbestos and other Toxic Particulates, University of Torino, Italy)
John Hadley (Owens Corning Science and Technology Center, Granville, OH, USA)
Tom Hesterberg (International Truck and Engine Corp., Warrenville, IL, USA)
Agnes Kane (Brown University School of Medicine, Providence, RI, USA)
David Lai (U.S. Environmental Protection Agency, Washington, DC, USA)
Ernest E. McConnell (ToxPath, Inc., Raleigh, NC, USA)
Hartwig Muhle (Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany)
Gunter Oberdorster (University of Rochester School of Environmental Medicine, Rochester, NY, USA)
Stephen Olin (ILSI Risk Science Institute, Washington, DC, USA)
David B. Warheit (DuPont Haskell Laboratory for Health and Environmental Sciences, Newark, DE, USA)

This working group report is the product of the joint efforts of the members of an expert working group organized and convened by the International Life Sciences Institute Risk Science Institute. All members of the working group contributed by drafting various sections of the report. The final report emerged from extensive discussions and revisions by the working group and represents the consensus of the group on the current utility and applicability of short-term assays and testing methods in screening fibers for potential toxicity. The screening strategy proposed by the working group is intended to distinguish between fibers that are unlikely to present a hazard and those that may require further testing for regulatory evaluation.

The ILSI Risk Science Institute expresses it sincere thanks to the members of this working group for their tireless efforts over many months and their collegial spirit in developing this report.

Financial support for this project through a cooperative agreement between the ILSI Risk Science Institute and the U.S. Environmental Protection Agency Office of Pollution Prevention and Toxics is gratefully acknowledged.

The International Life Sciences Institute (ILSI) is a nonprofit, worldwide foundation established in 1978 to advance the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment, and the environment for the well being of the general public. ILSI receives financial support from industry, government, and foundations.

The Risk Science Institute (RSI) was established in 1985 as part of the ILSI Research Foundation (RF) to improve the scientific basis of risk assessment. ILSI RF/RSI works toward this goal through an international program of scientific working groups, conferences and workshops, publications, and seminars. ILSI RF/RSI sponsors and participates in a wide range of activities to develop and disseminate new scientific knowledge, encourage exchange of ideas, and build consensus among scientists from academia, industry, government, and public interest/public health groups. ILSI RF/RSI and its programs are supported by government grants, cooperative agreements, and contracts, as well as by contributions from the ILSI Research Foundation and ILSI branches.

For further information on ILSI, RF, and RSI, see the ILSI web site: www.ilsi.org.

Received 12 January 2005; accepted 1 April 2005.
Address correspondence to Stephen S. Olin, International Life Sciences Institute Risk Science Institute, One Thomas Circle, NW, 9th Floor, Washington, DC 20005-5802, USA. E-mail: solin@ilsi.org

Table of Contents

EXECUTIVE SUMMARY

Purpose and Overview

Natural and synthetic fibrous materials are widely used in construction, industrial applications, and biomedical devices, as well as in consumer products. Fibrous materials are chemically and structurally diverse; however, for regulatory purposes, fibers are defined on the basis of their size and shape (aspect ratio $\geq$3:1, length $\geq$5 μm, and width $\leq$3 μm). Widely varied natural and synthetic fibers are currently in commercial use or under development, suggesting the need to understand the potential for adverse health effects if respirable fibers were released during production or application. In contrast to asbestos or synthetic vitreous fibers, other types of fibers have not been systematically assessed for carcinogenicity using lifetime rodent inhalation assays because these tests are technically demanding, expensive, and require large numbers of animals.

In 1996, the U.S. Environmental Protection Agency (EPA) added a "respirable fibers" category to the list of priority substances to be investigated for human health effects and exposure testing. In 2000 the U.S. EPA requested its FIFRA Scientific Advisory Panel to provide a peer review of current chronic carcinogenicity assays. The panel concluded that an updated assessment of the utility of short-term assays could greatly assist in prioritizing fibers for chronic assays.

This report summarizes the evaluation of short-term assays for fiber toxicity and carcinogenicity formulated by an expert working group convened by the ILSI Risk Science Institute. The objectives of the working group were:

1. To summarize the current state of the science on short-term assay systems for assessing potential fiber toxicity and carcinogenicity.
2. To offer insights and perspectives on the strengths and limitations of the various methods and approaches.
3. To consider how the available methods might be combined in a testing strategy to assess the likelihood that particular fibers may present a hazard and therefore may require further (e.g., long-term) testing for regulatory evaluation.

Health Effects of Fibers

Several types of pleural and parenchymal lung disease are associated with inhalation of asbestos fibers. Asbestos and erionite fibers have been classified by the International Agency for Research on Cancer (IARC) as carcinogenic for humans; refractory ceramic fibers and some special purpose glass microfibers have been classified by IARC as possible human carcinogens. Rodent models have proven to be appropriate surrogates for humans in reproducing the lung diseases associated with asbestos exposure. Although there are important anatomic and physiological differences that must be considered in extrapolating data from rodent models to humans, a lifetime chronic rodent inhalation study is considered to be the standard assay for assessing fiber carcinogenicity. Well-designed chronic rodent inhalation studies have been reported for a series of silica-based synthetic vitreous fibers (SVFs) and for a limited number of other types of fibers.

Respirability (the fraction of inhaled fibers reaching the alveolar region) is an important aspect of fiber pathogenicity. Respirability is determined by the aerodynamic diameter of the fiber, which is a function of diameter, length, and density. Surface charge and hydrophilicity, as well as adsorbed finishes and other physical and chemical factors, determine whether fibers can be easily dispersed or will agglomerate into larger, nonrespirable masses.

For respirable fibers tested in rodent bioassays, the dose, dimensions, durability in the lung, and in some cases surface reactivity of the fibers have been identified as critical parameters related to adverse health effects. Fiber length is hypothesized to be a major determinant of pathogenicity: Fibers that are too long to be completely phagocytized by macrophages are cleared less efficiently. If fibers are not rapidly leached or broken down in the lungs, long fibers have the potential to interact with other target cells in the lungs or be translocated to the interstitium or the pleura where they may cause disease. In chronic rodent inhalation studies, fibers that persisted in the lungs caused sustained inflammation and fibrosis. These pathologic endpoints were associated in most cases with the development of lung cancer or mesotheliomas in rodents after 1–2 yr. For SVFs, fiber length, diameter, and chemical composition are major determinants of biopersistence. Some organic fibers may also be degraded enzymatically in the lungs; however, there have been few studies of biopersistence of natural or synthetic organic fibers in rodents or humans.

Additional properties that have been linked to fiber toxicity, especially for natural crystalline fibers such as asbestos, include free radical generation, mobilization of transition metals, acquisition of iron, ferritin, or other proteins in the lungs, and surface hydrophilicity/hydrophobicity. At this time, no single physicochemical property or mechanism has been defined that can be used to predict carcinogenicity of all fiber types.

Current Testing Methods

Current short-term testing methods, defined as 3 mo or less in exposure duration, evaluate a number of endpoints that are considered relevant for lung diseases induced by fibers such as asbestos. Fiber durability can be estimated based on chemical composition and in vitro acellular or cellular dissolution assays. Biopersistence can be measured in short-term inhalation or intratracheal instillation studies. A variety of toxicologic endpoints can be measured in short-term, in vitro cellular assays (e.g., oxidant stress, release of proinflammatory mediators, genotoxicity). However, these in vitro cellular assays are not well standardized, and nonbiopersistent fibers may produce false positive results. Subchronic studies to assess biomarkers of lung injury (e.g., persistent inflammation, cell proliferation, fibrosis) are considered to be more predictive of carcinogenic potential. New approaches based on genomics and proteomics, and assays using genetically engineered mice may eventually lead to more sensitive, reproducible, validated assays to screen for potentially pathogenic fibers.

Proposed Testing Strategy for Prioritizing Fibers for Chronic Testing

The existing database of fiber toxicity studies strongly suggests that human exposure to respirable fibers that are biopersistent in the lung or induce significant and persistent pulmonary inflammation, cell proliferation, or fibrosis should be viewed with concern. The proposed testing strategy, using short-term assays to distinguish between fibers that are unlikely to present a hazard and those that may require further testing for regulatory evaluation, is based on this knowledge.

The proposed strategy has three fundamental components: preparation and characterization of an appropriate fiber sample, testing for biopersistence in vivo, and assessment of toxicologic endpoints in a subchronic rodent study.

1. *Preparation and characterization of fiber test sample:* The first step in short-term (or chronic) testing strategies is

derivation and characterization of a sample of long, respirable fibers from the bulk material for testing. The chemical composition, crystallinity and fracture habit, and size distribution of the bulk material and the size distribution and airborne concentrations of the fibers to which people are, or may be, exposed should be determined in the baseline characterization of the product. The sample of fibers to be tested should be respirable by the rodent species (preferably rat) with a significant proportion of fibers greater than 20 μm in length, at the same time reflecting the characteristics of fibers to which humans are exposed. The preparation of an appropriate fiber test sample is a critical and often challenging first step in fiber testing.

2. *Testing for biopersistence in vivo:* Biopersistence can be measured in a separate short-term rodent study (e.g., 5-day inhalation exposure), or in the subchronic rodent inhalation toxicity study by including subgroups for assessment of lung fiber burden after a 5-day exposure, at exposure termination (1–3 mo), and at the end of the postexposure recovery period. Standardized protocols, such as those developed in the European Union, are essential to obtain reliable and reproducible values for regulatory use. Fiber durability has been estimated by computer-based and in vitro methods, but these methods are not yet considered sufficiently robust for regulatory classification of fibers.
3. *Assessment of toxicologic endpoints in a subchronic rodent study:* The cornerstone of the testing strategy is a subchronic study in rodents (preferably rats), evaluating a range of toxicologic endpoints. The inhalation exposure route (at least 1 mo and preferably 3 mo exposure duration) is preferred, but intratracheal instillation is acceptable under certain conditions. Typically, a subchronic study will include at least two or three exposure concentrations, unexposed controls, and, periodically, controls exposed to other benchmark fibers (positive and negative). In a subchronic inhalation study, one exposure concentration of the test material should contain at least 150 fibers/cm^3 of fibers >20 μm in length. A recovery group should be included that, after termination of exposures, is held unexposed for at least 3 mo to determine if exposure-related effects persist. Key parameters to be evaluated in the subchronic study include lung weight and fiber burden, bronchoalveolar lavage (BAL) profile, cell proliferation, fibrosis, and histopathology. Other parameters may be added, depending on the fiber type.

The validity and utility of this testing strategy are well supported by the existing database for SVFs and asbestos fibers. For other fibers, an early indication of biological activity and potential hazard may be obtained using other short-term assays that, due to their technical limitations and lack of standardization, are not routinely included in the basic testing strategy. Particularly for non-SVF fibers (e.g., crystalline and surface-treated inorganic fibers), physicochemical assays for surface area and reactivity and short-term, in vitro cellular toxicologic assays may be useful to screen samples of fibers being considered for subsequent in vivo assays. Relatively few studies have been conducted on organic fibers that would permit an assessment of the applicability of specific short-term assay methods and the proposed testing strategy to this class of fibers.

1. PURPOSE

Natural and synthetic fibers are a group of substances of potential toxicological and public health concern. While many of these fibers have wide industrial and commercial applications, information regarding their potential impact on human health is often limited. As a result, a "respirable fibers" category has been added to the priority substances for health effects and exposure testing (U.S. EPA, 1996).

A workshop on chronic inhalation toxicity and carcinogenicity testing of respirable fibrous particles was cosponsored by the U.S. EPA National Institute of Environmental, Health Sciences (NIEHS), National Institute for Occupational Safety and Health (NIOSH), and Occupational Safety and Health Administration (OSHA) in 1995 (Vu et al., 1996). At that workshop, a number of short-term in vitro and in vivo assays were discussed that may be useful in assessing the relative potential of respirable fibers to cause lung effects. More recently, in September 2000, the U.S. EPA requested that the FIFRA Scientific Advisory Panel (SAP) provide a scientific peer review of its proposed guideline for evaluating the chronic toxicity and carcinogenic potential of fibers. Among the SAP's comments was a recommendation to update the state-of-the-science on short-term assay systems that might be available to assist in prioritizing fibers for testing and to inform the design of long-term studies.

In spring 2003 the ILSI Risk Science Institute (RSI) convened an expert working group to review and evaluate the available short-term assay systems for assessing fiber toxicity and carcinogenic potential. Short-term assay systems are important, at least in part, because the costs of carrying out chronic tests on all new and existing fibers are prohibitive. In addition, short-term assays may help to minimize the use of animals in toxicity testing. The objectives of the working group were:

1. To summarize the current state of the science on short-term assay systems for assessing potential fiber toxicity and carcinogenicity.
2. To offer insights and perspectives on the strengths and limitations of the various methods and approaches.
3. To consider how the available methods might be combined in a testing strategy to assess the likelihood that particular fibers may present a hazard and therefore may be candidates for further (e.g., long-term) testing.

This article, the product of the working group, provides an update of the state of the science and offers insights on the strengths and limitations of various approaches that may predict the long-term toxicity and carcinogenicity of respirable fibers. The strategy put forward is intended to describe the appropriate use of a combination of tiered short-term assays in assessing the potential for fiber toxicity and carcinogenicity and in prioritizing

fibers for long-term testing. It should facilitate delineation of fibers into those that are unlikely to present a hazard and those that warrant further investigation.

The existing data regarding fiber toxicity and carcinogenicity are based on chronic rodent assays using natural or synthetic inorganic fibers. The common endpoints used in these assays are biochemical markers of acute toxicity, persistent inflammation, fibrosis, and induction of lung cancer or malignant mesothelioma. Organic fibers may produce similar pathogenic effects, although very few organic fibers have been evaluated in chronic rodent assays. In addition, organic fibers have the potential to induce hypersensitivity or adaptive immune responses in the lungs. Evaluation of potential immunogenicity of organic fibers requires a different testing strategy than the short-term assays for toxicity and carcinogenity that will be presented in this article.

2. BACKGROUND

Fibers are useful and valuable materials for industrial and construction purposes and are in widespread use. However, the production and use of fibers and materials containing fibers produces the potential for release of respirable fibers that can be inhaled and could have adverse effects. Those exposed to asbestos represent by far the greatest population exposed to any respirable fiber type, and asbestos is still in use in huge amounts throughout the world. Following the epidemic of disease that arose from the mining and use of asbestos in the mid to late 20th century, there has been considerable growth and development in the use of other fiber types, both man-made and natural.

Fibers comprise a chemically and structurally heterogeneous group of materials that are defined on a regulatory basis by their shape. The World Health Organization (WHO, 1985) and NIOSH (1994) define fibers as particles with the following dimensions: length $>5\ \mu m$, width $<3\ \mu m$, aspect ratio $>3{:}1$. This definition is not health based and takes no account of composition, which is a key factor in understanding differences in the pathogenicity of fibers. [Note also that the standard phase contrast optical microscopy (PCOM) method has a limit of detection of approximately $0.3\ \mu m$; fibers thinner than this can only be detected by electron microscopy.] Respirable fibers are those that can reach the alveolar region upon inhalation.

The broad range of fiber types in use and the continued development of new fiber types with new properties are a challenge for the regulatory toxicology community. Figure 1 outlines some of the different fiber types that have been considered for their health effects; the list is not exhaustive but intends to demonstrate the heterogeneity of fiber types that are available. The shaded area of Figure 1 encompasses the group of fiber types known as silica-based synthetic vitreous fibers (SVFs). SVFs, also called

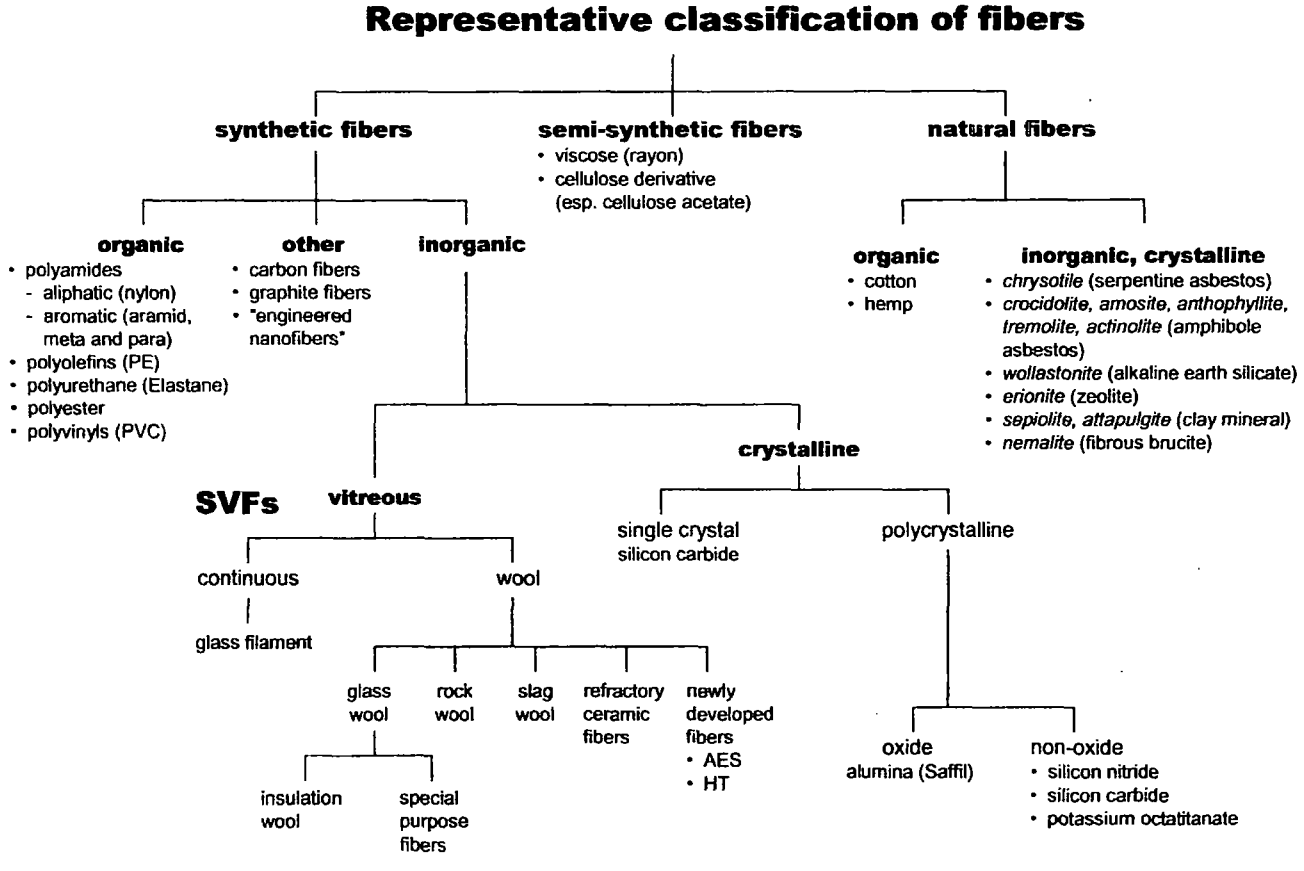


FIG. 1. Representative classification of fibers.

man-made vitreous fibers (MMVFs) or manufactured vitreous fibers (MVFs), are noncrystalline (vitreous) materials manufactured primarily from glass, rock, minerals, slag, and processed inorganic oxides. The range of chemical and physical properties of SVFs, their production methods, and their uses have been summarized recently (IARC, 2002).

3. HEALTH EFFECTS OF FIBERS

Several types of pleural and parenchymal lung disease are associated with inhalation of asbestos fibers (Table 1). Animal models have proven to be appropriate surrogates for humans for defining the various disease entities associated with asbestos exposure. For many of these diseases, there is a correlation between the type of asbestos exposure, the intensity and duration of exposure, and the severity of the disease. Pleural plaques and fibrosis (including pleural pseudotumors or rounded atelectasis) are markers of asbestos exposure but are not causally related to lung cancer or diffuse malignant mesothelioma. Fibrotic scarring of the pleura or lung parenchyma is a nonspecific reaction to chronic inflammation or trauma; however, bilateral, symmetrical pleural plaques usually indicate occupational or environmental exposure to asbestos fibers or erionite (Travis et al., 2002). Asbestosis or diffuse interstitial fibrosis may progress after cessation of exposure and is considered a risk factor for development of lung cancer by some investigators (Churg & Green, 1998) but not by others (Nelson & Kelsey, 2002). The incidence of lung cancer is greatly increased in cigarette smokers or ex-smokers who are also exposed to asbestos fibers. Diffuse malignant mesothelioma arising in the pleura or peritoneum occurs less frequently and after a longer latency period than pleural fibrosis or asbestosis; it can develop in the absence of asbestosis or cigarette smoking and occurs more frequently in people exposed to amphibole asbestos fibers or erionite. The association between asbestos exposure and carcinoma of the larynx or gastrointestinal tract is controversial (Churg & Green, 1998).

TABLE 1
Diseases associated with exposure to asbestos fibers

Disease	Human	Animal models
Nonneoplastic pleural changes		
Pleural effusion	+	+
Visceral pleural fibrosis	+	+
Parietal pleural plaques	+	+
Asbestosis (diffuse interstitial fibrosis)	+	+
Carcinoma of the lung	+	+
Malignant mesothelioma of the pleura and peritoneum	+	+
Gastrointestinal carcinoma	+/−	+/−

Note. Adapted from Churg and Green (1998), animal data from Hesterberg et al. (1993), Mast et al. (1995), McConnell (1994), and McConnell et al. (1999). +/−, Conflicting reports.

An unusual form of interstitial pulmonary response that may have an immunopathological component has been described in workers exposed to nylon flock fibers (Warheit et al., 2001a; Kern et al., 2000).

Crocidolite, chrysotile, tremolite, amosite, actinolite, and anthophyllite asbestos have been evaluated by IARC as proven (Group 1) human carcinogens (IARC, 1987). IARC has also evaluated glass wool, continuous glass filament rock wool/stone wool and slag wool (IARC, 2002), *para*-aramid fibrils, and wollastonite fibers (IARC, 1997) as having inadequate evidence of carcinogenicity in humans (Group 3) but has classified refractory ceramic fibers and special-purpose glass fibers (such as E-glass microfibers) as possible human carcinogens (Group 2B) (IARC, 2002) (Table 2).

Glass wools and rock/slag wools have shown no carcinogenic effects in humans or rodents but inflammatory effects have been documented at high exposure. Refractory ceramic fibers (RCFs) have produced lung cancer and mesotheliomas in rats, and RCF has also produced mesotheliomas in hamsters. There are also reports of pleural plaques in some human populations exposed to RCFs, but no mesotheliomas have been reported.

In addition to fibers that have been evaluated by IARC, silicon carbide whiskers have induced lung tumors after inhalation in rats (Lapin et al., 1991; Miller et al., 1999). Induction of mesothelioma was observed after intrapleural inoculation (Johnson & Hahn, 1996), and after intraperitoneal injection a high incidence of abdominal mesotheliomas was observed (Pott et al., 1991). The pulmonary response after intratracheal instillation in Fischer rats showed chronic inflammation and the development of pulmonary granulomas in a study up to 18 mo (Vaughan et al., 1993). An excess risk of lung cancer was observed in a study in the Norwegian silicon carbide industry, although other potentially carcinogenic dusts may have contributed (Romundstad et al., 2001). Also, 3-mo aerosol exposures to potassium octatitanate fibers produced pleural mesotheliomas in a few hamsters at 18 mo postexposure (Lee et al., 1981).

The pathologic reactions associated with inhalation of weakly toxic dusts (e.g., silicates) include dust macules and small airway fibrosis in both humans and animals. Nodular or diffuse interstitial fibrosis that may extend to the visceral pleura is characteristic of highly toxic dusts or fibers (e.g., crystalline silica, asbestos). Foreign-body granulomas are characteristic of pneumoconiosis induced by silicates; rarely, these granulomas may coalesce to nodular or diffuse fibrosis. Foreign-body giant cells are occasionally seen in association with asbestos bodies (Churg & Green, 1998). In general, the pathologic changes characteristic of silicate pneumoconiosis (e.g., from talc not contaminated with asbestos fibers) are less severe than asbestosis, and these minerals are not classified as known or possible human carcinogens (Table 2). Rat inhalation studies using amosite or chrysotile asbestos fibers in combination with crystalline silica or titanium dioxide appeared to enhance translocation of fibers to the pleura and produced more mesotheliomas

TABLE 2
IARC evaluations of fibers

Fiber	Overall evaluation	Group
Asbestos (actinolite, amosite, anthophyllite, chrysotile, crocidolite, tremolite)	Carcinogenic to humans	Group 1
Erionite	Carcinogenic to humans	Group 1
Refractory ceramic fibers	Possibly carcinogenic to humans	Group 2B
Special purpose glass fibers (e.g., E-glass microfibers)	Possibly carcinogenic to humans	Group 2B
Glass wool	Not classifiable as to their carcinogenicity to humans	Group 3
Continuous glass filament	Not classifiable as to their carcinogenicity to humans	Group 3
Rock (stone) wool	Not classifiable as to their carcinogenicity to humans	Group 3
Slag wool	Not classifiable as to their carcinogenicity to humans	Group 3
para-Aramid	Not classifiable as to their carcinogenicity to humans	Group 3
Wollastonite	Not classifiable as to their carcinogenicity to humans	Group 3
Palygorskite (attapulgite)		
Long fibers (>5 μm)	Possibly carcinogenic	Group 2B
Short fibers (<5 μm)	Not classifiable	Group 3
Sepiolite	Not classifiable	Group 3

Note. Data from IARC (1987, 1997, 2002).

than amosite or chrysotile asbestos alone (reviewed by Davis, 1996).

Organic Fibers. Occupational exposure to natural organic fibers, such as cotton, flax, hemp, and cellulose, has been documented. Exposure to cotton or flax dusts has been associated with airway obstruction, airway hyperreactivity, and declines in cross-shift dynamic lung volumes (Merchant et al., 1972; Jacobs et al., 1993). The etiologic agent in these dusts appears to be endotoxin rather than the natural organic fibers themselves (Castellan et al., 1984, 1987). Indeed, animal inhalation exposure to endotoxin-free cellulose appears to cause minimal pulmonary response (Fischer et al., 1986). Pulmonary fibrosis is not a common consequence of cotton dust exposure (Rylander et al., 1987), and cotton textile workers exposed to cotton dust have a lower than normal incidence of lung cancer (Lange, 1988).

Synthetic organic fibers (SOFs) have been produced for over 50 yr. Although some chemicals used in the production of these fiber types have been investigated for adverse health effects in occupationally exposed people there is a limited toxicological database regarding the pulmonary effects of inhaled SOF dust. This stems, in large part, from two perceptions: (1) Occupational exposures to airborne SOFs are generally very low; (2) SOF dust in the workplace was assumed to be nonrespirable (i.e., not small enough to deposit in the distal (gas exchange) regions of the lung). Thus, it was assumed that occupational exposures to respirable SOFs were not of concern. However, as SOFs are being adapted for an increasing variety of applications, thinner fibers are being produced, and newer processing techniques (such as chopping or flocking) have resulted in significant levels of respirable airborne dust and, in some cases, pulmonary disease.

For example, nylon flock worker's lung is an interstitial inflammatory disease characterized by a prominent lymphocytic infiltrate and lymphoid follicles with germinal centers surrounding bronchioles and alveolar ducts (Eschenbacher et al., 1999).

In contrast, asbestosis is characterized by patchy interstitial fibrosis that is more severe in the subpleural areas of the lower lobes and accompanied by a mild lymphocytic infiltrate in most cases (Travis et al., 2002). Organic fibers may trigger adaptive immune or hypersensitivity responses in susceptible or sensitive human populations (Eschenbacher et al., 1999).

Because much is known about the determinants of biological activity of silicate fiber types, and relatively little is known about the activity of SOFs, it is tempting to assume that what has been learned about the former also applies to the latter. However, it is important to recognize that the following questions remain unanswered for SOFs: Do the three D's—dose, dimension, and durability (see section 5.1)—apply to SOFs? Are other factors that are less important for SVF toxicology of greater importance in determining the biological activity of SOFs—such as surface area, chemical composition, surface activity, hydrophilicity/hydrophobicity, antigenicity, and adsorbed finishes, dyes, and other additives? Are the pulmonary responses to SOFs similar to inorganic fiber types (i.e., activation of signal transduction pathways and cytotoxic factors upon cell-contact with fibers, macrophage responses and lung inflammation; in some cases fibrosis, and cancer)? Are the cellular and molecular responses to SOFs qualitatively different from those of silicate fiber types? Have immunological considerations been adequately evaluated?

Respirability of fibers is primarily determined by aerodynamic diameter, which is in turn determined by size, shape, and density. SOFs are less dense and can be more curly than SVFs. Furthermore, SOFs tend to have charged surfaces. This creates challenges for air sample collection, as the charged

fibers tend to adhere to the sides of air sample collection devices.

4. PROPOSED MECHANISMS OF FIBROGENIC AND CARCINOGENIC EFFECTS

Pathogenicity of fibers is a complex issue that depends on multiple factors, including fiber dimensions; these factors are discussed in depth in section 5.3. Fibers have an aerodynamic diameter that is 1.5 to 3 times their actual diameter depending on their density and length (Oberdorster, 1996), and even very long fibers can penetrate deeply into the lungs, provided that they are thin. Long, thin fibers can penetrate beyond the ciliated airways to where they present the alveolar macrophage system with problems of effective phagocytosis and clearance. Slow clearance of long biopersistent fibers is well documented (Coin et al., 1994; Hesterberg et al., 1998a; Searl et al., 1999). Short fibers can be effectively phagocytosed by macrophages, but under high exposure conditions short biopersistent fibers can overload clearance mechanisms and may be pathogenic. Macrophages have difficulty completely engulfing long fibers and frustrated or partial phagocytosis can result in chronic stimulation of the macrophages and failure of clearance. Fibers that penetrate beyond the ciliated airways can interact with local target cells or translocate to other sites, especially to the interstitium and the pleura, causing inflammation and fibrosis that are potentially important processes in carcinogenesis.

Several mechanisms have been proposed for the fibrogenic and carcinogenic effects of fibers (Tables 3 and 4). Most of these mechanistic studies have used inorganic, crystalline fibers, and whether these same mechanisms operate for all fibers is unknown.

TABLE 4
Indirect mechanisms of asbestos fiber carcinogenesis

Mechanisms	References
Cofactor with cigarette smoke	Reviewed in Kane (1996); Lee et al. (1998); Nelson and Kelsey (2002)
Cofactor with SV40 virus	Reviewed in Gazdar et al. (2002)
Persistent inflammation with secondary genotoxicity	Vallyathan and Shi (1997)
Persistent inflammation with release of cytokines and growth factors	Reviewed in Brody et al. (1997)

Note. Adapted from *IARC* (1999).

4.1. Direct Effects

4.1.1. Fiber-Derived Free Radicals Damage DNA

There is abundant evidence that asbestos fibers (Hardy & Aust, 1995) and some synthetic fiber types (Gilmour et al., 1997; Donaldson et al., 1996; Pezerat et al., 1989) can generate free radicals by different mechanisms, including hydroxyl radical, by Fenton chemistry. It is hypothesized that these radicals can form DNA adducts such as 8-hydroxy-deoxyguanosine (8-OH-DG) (Hardy & Aust, 1995; Fubini & Otero-Arean, 1999) that are misrepaired giving rise to mutations. This could potentially

TABLE 3
Direct mechanisms of asbestos fiber carcinogenesis

Mechanism	Experimental end-points	References
Genotoxic	Oxidized bases	Chao et al. (1996); Fung et al. (1997)
	DNA breaks	Reviewed in Jaurand (1996)
	Aneuploidy	Reviewed in Jaurand (1996); Jensen et al. (1996)
	Mutations	Park and Aust (1998)
	Deletions	Reviewed in Hei et al. (2000)
Nongenotoxic		
Mitogenic	Target cell proliferation	BéruBé et al. (1996); Goldberg et al. (1997)
	Binding to or activation of surface receptors	Boylan et al. (1995); Pache et al. (1998)
	Growth factor expression	Liu et al. (1996); Brody et al. (1997)
	Activation of signaling pathways	Reviewed in Mossman et al. (1997); Manning et al. (2002)
Cytotoxic	Apoptosis	Broaddus et al. (1996); Goldberg et al. (1997); Levresse et al. (1997)
	Necrosis	Reviewed in Kane (1996)

Note. Adapted from *IARC* (1999).

occur in any target cell that made contact with the fiber. Asbestos has been reported to cause mutations resulting from large deletions ranging in size from a few thousand to several million base pairs (Hei et al., 1995). Asbestos has also been reported to cause direct transformation of cells in vitro that developed through sequential steps, including altered growth kinetics, resistance to serum-induced terminal differentiation, and anchorage-independent growth, and were finally tumorigenic in nude mice (Hei et al., 2000).

Recent in vivo studies have confirmed some of these in vitro genotoxicity endpoints. For example, lipid (Ghio et al., 1997) and hydroxyl radicals have been measured after intratracheal instillation of asbestos fibers in rat lungs (Schapira et al., 1994). Two independent studies have found increased mutation frequency at the *lacI* reporter gene locus in response to crocidolite asbestos after inhalation (Rihn et al., 2000) or intraperitoneal injection (Unfried et al., 2002). The latter study also found increased levels of 8-OH-dG in DNA extracted from omentum after intraperitoneal injection of crocidolite asbestos fibers in rats.

4.1.2. *Fibers Interfere With Mitosis*

Fibers have been reported to directly interact with the mitotic spindle and chromosomes during mitosis (Hesterberg et al., 1985) in in vitro studies. This could lead to aneuploidy, polyploidy, binucleate cells, and micronucleus formation (Kane, 1996; Dopp et al., 1995; Ault et al., 1995), all of which are relevant for carcinogenesis as confirmed by the fact that these changes have been found in cell lines derived from human and animal mesotheliomas (reviewed in Kane, 1996).

4.1.3. *Fibers Directly Stimulate Proliferation of Target Cells*

Several studies have demonstrated the ability of asbestos fibers to stimulate cell proliferation. This has been demonstrated following in vitro exposure of tracheobronchial epithelial cells (Sesko et al., 1990), fibroblasts (Lasky et al., 1996), and pleural mesothelial cells (Heintz et al., 1993). Proliferation has also been observed following in vivo exposure in the mesothelial (Adamson et al., 1993) and airspace epithelial compartments (Brody et al., 1987). Several mechanisms have been identified for the proliferative effects of fibers: direct activation of growth factor receptors, increased expression of growth factors, activation of intracellular signaling pathways, and compensatory cell proliferation in response to apoptosis or necrosis (summarized in Table 3).

4.2. Indirect Effects

4.2.1. *Asbestos and Cigarette Smoke*

Epidemiological evidence suggests that cigarette smoking and asbestos produce a multiplicative risk for lung cancer (Kjuss et al., 1986). Asbestos has a high surface area that may facilitate adsorption of carcinogens (Lakowicz et al., 1980) and their delivery to tissues that they might not otherwise reach. Several studies have demonstrated that polycyclic aromatic hydrocarbons (PAHs) bind to fibers (Lakowicz & Bevan, 1979) and are then made available to microsomes, where they can be activated (Kandaswami & O'Brien, 1983). PAHs and asbestos together were more potent in causing squamous metaplasia in vitro than either substance alone (Mossman et al., 1984). Oxidants released from inflammatory cells or directly catalyzed by fibers may exacerbate tissue injury and regeneration triggered by cigarette smoke. Asbestos fibers have been proposed to enhance chromosomal instability and mutations in the *K-ras* oncogene and the *p53* tumor suppressor gene in cigarette smokers (reviewed in Nelson & Kelsey, 2002). Alternatively, genetic or acquired alterations in DNA repair pathways may contribute to the increased risk of lung cancer in cigarette smokers or ex-smokers exposed to asbestos fibers (Hu et al., 2002; Hartwig, 2002).

4.2.2. *SV40 Virus and Malignant Mesothelioma*

SV40 virus has been proposed as a cofactor with asbestos in the induction of malignant mesothelioma in humans but is highly controversial (reviewed in Gazdar et al., 2002). SV40 virus has also been found in spontaneous human osteosarcomas, as well as in brain and pituitary tumors. Millions of people who received polio vaccines in the 1950s and 1960s were inadvertently exposed to SV40 virus, and it has been proposed that the virus is transmitted vertically. SV40 viral T-antigen has been detected in 60–80% of human malignant mesothelioma samples and cell lines, although the sensitivity and specificity of this association is currently controversial.

4.2.3. *Fibers Provoke a Chronic Inflammatory Reaction Leading to the Prolonged Release of Reactive Species (ROS/RNS), Cytokines, and Growth Factors*

Macrophages phagocytosing long fibers can be damaged or can release mediators that cause inflammation, such as cytokines, or those that cause bystander tissue damage, such as oxidants and proteases. Increased macrophage stimulatory or cytotoxic activity of long fibers has been demonstrated in vitro using a range of fiber types (Brown et al., 1986), long and short amosite asbestos fibers (Donaldson et al., 1992), and size-fractionated glass fiber preparations (Ye et al., 1999). Ineffective phagocytosis and clearance of long fibers may also result in fibers interacting with epithelial cells for protracted periods, which could stimulate them to release proinflammatory chemokines (Luster & Simeonova, 1998). Macrophages and polymorphonuclear leukocytes (PMN) recruited to the lungs can release an array of oxidants and mitogens that could cause proliferation (Robledo et al., 2000) and produce adducts, oxidized bases, single- and double-strand breaks, and DNA cross-links (Driscoll et al., 1997). These lesions are potentially mutagenic if they are not accurately repaired.

Inhalation of asbestos or erionite fibers at high doses in humans causes diffuse interstitial fibrosis or asbestosis that is usually more prominent in the lower lobes of the lungs. This fibrotic reaction develops slowly but progressively, beginning 10 yr after the initial exposure. It is hypothesized that oxidants and proteases released from alveolar macrophages activated by phagocytosis of fibers damage the alveolar epithelial lining. In addition, fibers may become translocated across the damaged epithelium into the interstitium of the alveolar walls. This injury can be repaired by a combination of epithelial regeneration by Type II alveolar cells plus proliferation of fibroblasts with collagen deposition in the interstitium. Upregulation of growth-factor expression has been observed at sites of asbestos fiber deposition in rat lungs: Platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β are hypothesized to trigger fibroblast proliferation and collagen synthesis, respectively, while TGF-α is mitogenic for alveolar epithelial cells (reviewed in Brody et al., 1997). Fibers also translocate to the pleural space following inhalation and accumulate near lymphatic openings on the parietal pleura and the dome of the diaphragm (Boutin et al., 1996); these are the anatomical sites where fibrotic or calcified pleural plaques develop. Pleural plaques are considered as a marker of prior asbestos exposure; they can occur even in the absence of asbestosis. Diffuse fibrosis of the visceral pleura can also occur, usually following repeated episodes of pleural effusion that is also called benign asbestos pleurisy. It is hypothesized that asbestos-induced pleural effusions are caused by release of chemokines such as interleukin (IL)-8 from mesothelial cells (Boylan et al., 1992). Pleural fibrosis and pleural plaques are hypothesized to develop after injury to mesothelial cells and destruction of the basement membranes. This injury is then repaired by mesothelial and submesothelial cell proliferation and deposition of collagen (Davila & Crouch, 1993).

Recent experimental evidence based on animal models has provided new insight about the mechanistic basis for the association of chronic inflammation and fibrosis with cancer (Coussens & Werb, 2003). Recruitment and activation of inflammatory cells in response to persistent infection or asbestos fibers are accompanied by release of reactive oxygen and nitrogen species that could damage DNA, induce oxidant stress, and lead to mutations. Chronic inflammation is frequently accompanied by increased epithelial cell turnover and type II cell hyperplasia in the lung (Travis et al., 2002). Cytokines and growth factors derived from inflammatory cells may contribute to proliferation of preneoplastic cells; proteases released from activated stromal cells may increase extracellular matrix turnover and facilitate invasion of tumor cells (Tlsty, 2001).

5. CHEMICAL AND PHYSICAL CHARACTERISTICS OF FIBERS POTENTIALLY RELEVANT TO HEALTH EFFECTS

5.1. Introduction

Not all fibers, as defined by the WHO/NIOSH, are of equal pathogenic potency. The likelihood that any airborne fiber sample will cause pathogenic effects depends on the crystallinity and chemical composition of the fibers—which covers a wide variety types (Figure 1)—and on their diameter, length and biopersistence.

For an evaluation of the interactions of airborne fibers inhaled by humans, it is useful to consider exposure–dose-response relationships (Figure 2). The term *exposure* should not be confused with *dose*: The former can be expressed as an airborne concentration (fiber number/cm^3; μg/m^3), whereas the latter refers to the amount of fibers actually retained in the different regions of the respiratory tract. Figure 2 shows specific parameters associated with exposure with respect to the sources, physicochemical properties, concentrations of fibers, and activities of humans. The dose retained after inhalation of fibers depends on the deposition and clearance/retention of the fibers, which can be expressed as number, surface area, or mass (dose metric), and the dimension of the fibers in terms of their lengths and diameters. Ensuing responses, conditioned by individual susceptibilities, include inflammatory, fibrotic, and carcinogenic endpoints, with the last consisting of lung tumors and mesotheliomas. The following paragraphs focus on the dose parameters outlined in Figure 2, since they are of importance for hazard identification and risk assessment. Dose, dimension and durability (the three "D's") of fibers are the most important parameters for many

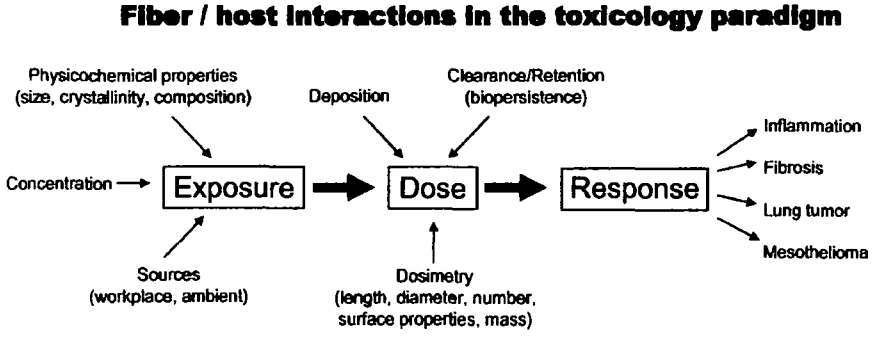


FIG. 2. Airborne fibers and host interactions.

fiber types, including SVFs, and are part of the individual factors shown in Figure 2.

5.2. Deposition of Inhaled Fibers

The main deposition mechanisms of inhaled fibers—like those for spherical particles—include impaction (when there are abrupt directional changes in the airways), sedimentation (due to settling of airborne fibers by gravitational forces), and diffusion (due to the movement of surrounding air molecules [Brownian motion]). Although the first two mechanisms apply to larger particles with aerodynamic diameters $>\sim 0.5\ \mu m$, the third mechanism, diffusion, becomes increasingly more important for very small fibers with aerodynamic diameters equivalent to unit density spherical particles $<0.5\ \mu m$ in diameter. A fourth mechanism affects mainly longer fibers, that is, deposition via interception when the ends of the fiber moving in an airstream contact airway walls resulting in deposition of the fiber. This mechanism results in an effective filtration of long fibers in the nose, which is an important factor limiting the respirability of long fibers in rodents as obligatory nose breathers.

When rodents and humans are exposed to the same concentration of fibers of the same size distribution, the dose depositing in their respective lower respiratory tracts will be quite different since fiber respirability differs significantly between these two species. Respirability is defined as the fraction of inhaled fibers reaching the alveolar region. Although qualitatively the shape of fiber deposition curves in the lung are similar between rats and humans, the aerodynamic diameters of both fibrous and nonfibrous particles that will reach the alveolar structures of the rodent and human lung are very different. There is a theoretical upper limit aerodynamic diameter of $\sim 3\ \mu m$ for fibers reaching the centriacinar region in the rat lung and $\sim 6\ \mu m$ in the human lung (Dai & Yu, 1998; Oberdorster, 1996).

5.3. Aerodynamic Diameter and Fiber Dimensions

The parameter "aerodynamic diameter" is very important when describing the movement and deposition efficiency of inhaled fibrous and nonfibrous particles. For any particle, the aerodynamic diameter of an irregularly shaped particle is equivalent to the geometric diameter of a sphere of unit density that has the same terminal settling velocity in still air as the particle in question. With respect to fibers, this relationship between geometric diameter and aerodynamic diameter depends on both fiber diameter and fiber length, whereby the diameter has a much greater influence. Specific density of the fiber plays a role as well. Aerodynamic principles of particle deposition in the lungs predict that thicker, higher density fibers will be less respirable and either will not enter the lower respiratory tract or will deposit in the upper airways to be effectively cleared by the mucociliary escalator. A fiber with a diameter of 3 μm is classified as a regulated fiber by WHO/NIOSH but would deposit with very low efficiency in the region of the human lung beyond the ciliated airways, as the deposition curves show that this fiber is at the borders of respirability. This contrasts with fibers of 0.1 μm aerodynamic diameter that deposit with optimal (around 50%) efficiency; similar considerations apply in the rat (Oberdorster, 1996). Density affects aerodynamic diameter but has not been considered as an important variable, since the most studied fibers, asbestos and the SVFs, have a density that is 2–3 g/cm^3; however, organic fibers may have densities of less than 1 g/cm^3, which would increase their respirability.

Stanton, using implantation of fibers into the rat pleura, and Pott, using intraperitoneal injection, identified that very short fibers were less pathogenic as measured by their ability to cause "pleural sarcomas" or peritoneal mesothelioma. Stanton also suggested that fibers longer than about 8 μm were more carcinogenic than shorter fibers (Stanton et al., 1981). Davis and coworkers demonstrated that long fiber amosite was highly carcinogenic by inhalation and that the same mass exposure to a preparation of the same fibers, dramatically shortened by milling, produced virtually no tumors or fibrosis (Davis et al., 1986); however, subsequent studies showed some surface differences between the original and the ground material (Gilmour et al., 1995; Hill et al., 1995). Muhle et al. (1987) also found short-fiber crocidolite not to be carcinogenic after inhalation. A range of fibers of different composition were studied for their ability to cause pathology in the Swiss RCC studies, and analysis of these data indicated that extent of biopersistence and proportion of fibers longer than 20 μm were the best descriptors of the ability of any inhaled fiber preparation to cause tumors in rats (Bernstein et al., 2001a, 2001b). In Scotland the Colt fiber program analyzed the RCC data plus data from their own studies with different fibers and came to the same conclusion (Miller et al., 1999). In further confirmation of the role of fiber length, inhalation studies by the National Institute of Environmental Health Sciences (National Institutes of Health, NIH) showed that a long-fiber chrysotile asbestos sample was carcinogenic (McConnell et al., 1984), whereas the same mass concentration of short-fiber-length chrysotile was not (Ilgren & Chatfield, 1998).

5.4. Clearance of Deposited Fibers

In general, the dose of an inhaled compound retained in the respiratory tract at any time is equivalent to the deposited dose minus the amount cleared. For poorly soluble compounds, including fibers, several physiological clearance mechanisms contribute to their elimination from lung, which include (Figure 3):

- Movement by the mucociliary escalator in the nose and tracheobronchial region.
- Phagocytosis by alveolar macrophages in the alveolar region (this is limited to fibers that can be phagocytized by macrophages, i.e., long fibers are not subjected to this clearance mechanism; in rats the normal macrophage-mediated clearance of poorly soluble particles of low cytotoxicity occurs with a retention halftime of about 70 days, whereas in humans this retention halftime is between 400 and 700 days).

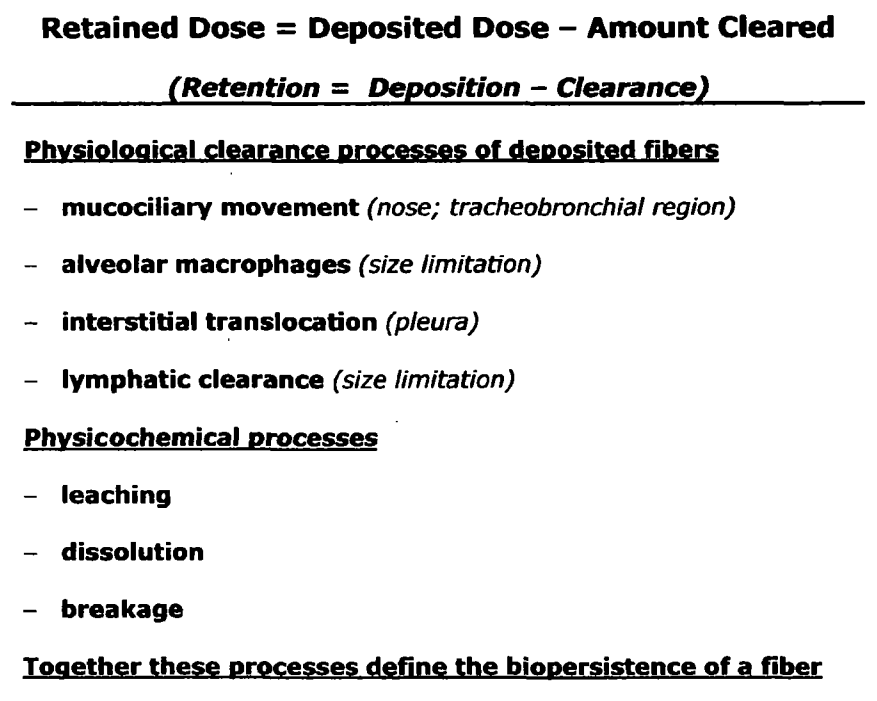


FIG. 3. Biopersistence of a fiber.

- Interstitial translocation of deposited fibers, including translocation to the pleural sites, which appears to be more efficient for short fibers (Gelzleichter et al., 1999).
- Clearance via lymphatic channels once fibers have reached the interstitium (this pathway also is limited by size with a maximal fiber length of ~9 μm; Oberdörster et al., 1988).

In addition to these physiological clearance mechanisms, specific physicochemical processes (leaching, dissolution, breakage) will also contribute to the elimination of fibers, and together these mechanisms define the biopersistence of a fiber in the respiratory tract (see section 5.5).

With respect to defining a threshold of fiber length below which fibers are less pathogenic, it is hypothesized that phagocytosis by alveolar macrophages is a decisive factor. Fibers deposited in the lung that are too long to be completely phagocytized by alveolar macrophages are less likely to be cleared out of the alveolar compartment unless they are biosoluble. Thus, they can interact with epithelial cells, may become interstitialized, and are more likely to be transported to pleural sites than short fibers which are readily phagocytized by alveolar macrophages. A limiting factor for alveolar macrophage phagocytosis is the diameter of the macrophages in the alveolar space, and respective values have been reported to range between 10.5 and 13 μm for the rat and between 14 and 21 μm for humans (Crapo et al., 1983; Lum et al., 1983; Stone et al., 1992; Sebring & Lehnert, 1992; Krombach et al., 1997). This size limitation for effective macrophage-mediated clearance has to be considered when extrapolating from rodent fiber inhalation studies to humans. Although these numbers should not imply that fibers longer than the diameter of a macrophage cannot be phagocytized—alveolar macrophages certainly can phagocytize fibers longer than their diameter by adapting their shape—they indicate those fiber length categories that may be most pathogenic. The concept of long fiber pathogenicity has been emphasized by regulatory agencies (e.g., EC Directive on Classification of Synthetic Vitreous Silicate Fibers) and at scientific meetings (Vu et al., 1996). Primarily, fibers longer than approximately 15 μm in length should be considered with respect to a tumorigenic potential, and special attention should be given to their determination in animal studies evaluating biopersistence of newly developed fibers. It appears, however, that for noncancer endpoints the number of all fibers should be considered, since even completely phagocytized fibers result in activation of alveolar macrophages and will contribute to an increase in the degree of an inflammatory response.

5.5. Biopersistence

The Existing Database. A fiber is biopersistent when it remains in the lungs despite physiological clearance, translocation and dissolution/breakage. Most of our knowledge of the role of biopersistence as it relates to the process of carcinogenesis is based on a number of large studies where a range of fibers were tested in animal studies, principally the RCC studies, but also the IOM/Colt studies (see Table 5). Most of the fibers studied were noncrystalline SVFs, with asbestos as a positive control. Thus, the database on the links between in vitro biopersistence,

TABLE 5
General scheme relating biopersistence and pathogenicity of natural and synthetic fibers in chronic rodent inhalation assays

Fiber type	Biopersistence	Persistent inflammation	Fibrosis	Lung cancer	Mesothelioma
Chrysotile asbestos	+/−	+	+	+	+
Crocidolite asbestos	+	+	+	+	+
Amosite asbestos	+	+	+	+	+
Refractory ceramic fiber (RCF1)	+	+	+	+	+
Special-purpose glass fibers (e.g., E-glass microfibers)	+	+	+	+	+
Rock (stone) wool	+/−	+/−	+/−	−	−
Glass wool	−	−	−	−	−
Slag wool	−	−	−	−	−
Wollastonite	−	−	−	−	−
para-Aramid fibrils	−	+/−	+/−	−	−

Note. Specific fiber chemical compositions may fall into different categories depending on their individual characteristics. From McConnell et al. (1984, 1991; McConnell, 1994), Warheit (1995), Bernstein et al. (2001a), and IARC (2002).

in vivo biopersistence, and pathologic effects is derived from this limited class of silica-based synthetic vitreous fibers. The extent to which this understanding of biopersistence that we have gained for these fibers is generalizable to other fibers (e.g. organic fibers, crystalline fibers, nonoxide fibers) is not known, and more research is needed before we use the same paradigm for these other fiber types.

The Effects of the Lung Milieu on Fibers. Fibers vary in their structural response to residence in the milieu of the lung. The chemical structure of some fibers renders them wholly or partially soluble, and such fibers are likely to either dissolve completely, or dissolve until they are sufficiently weakened focally to undergo breakage into shorter fibers. Short fibers are then likely to undergo successful phagocytosis and clearance by the macrophage system (Figure 4).

Fiber dissolution in the lung could occur both intracellularly and extracellularly. In either scenario there is a decrease in the number of long fibers. Chrysotile asbestos contains a "Brucite" layer of magnesium hydroxide in its "carpet roll" structure, and this readily leaches, contributing to the relatively lower biopersistence of chrysotile (Morgan, 1997) compared to the amphiboles, which have no such leachable weak point in their structure. Studies with man-made fibers have identified that biopersistence is a major factor in determining the ability to cause pathological effects in animals in long-term studies (Hesterberg et al., 1998a, 1998b; Miller et al., 1999; Oberdorster, 2000). In vivo (Bellmann & Muhle, 1994; Muhle & Bellmann, 1995) and in vitro (Hesterberg et al., 2002) assays have been developed to predict long-term in vivo biopersistence. However, in vitro assays do not subject the fibers to the full spectrum of influences that could determine biopersistence in the lungs and are better described as measures of durability that can be used to extrapolate biodurability and biopersistence. Nevertheless, in the absence of other information, these assays may indicate whether a given fiber is likely to cause disease if inhaled.

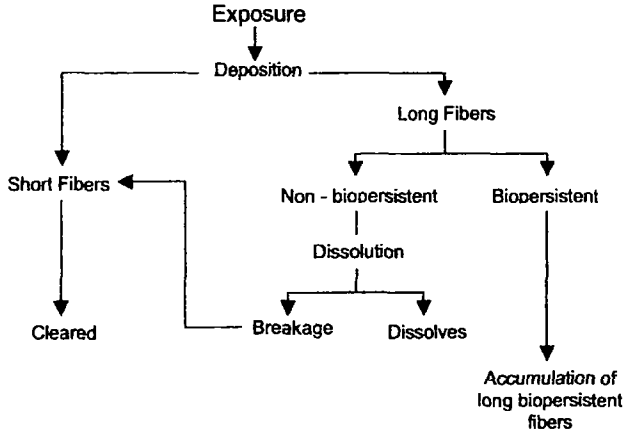


FIG. 4. Disposition and fate of fibers in the lung.

Some organic fibers appear to also be susceptible to degradation through an enzymatic mechanism (Hesterberg et al., 2000). There is little data available on the biopersistence of other organic fibers. In one study the durability of cellulose fibers in rat lungs was investigated and the cellulose fibers were found to be more biopersistent than ceramic fibers (Muhle et al., 1997). Inhalation studies with *p*-aramid respirable-sized, fiber-shaped particulates (RFP) have demonstrated that the respirable fibers that deposit in the alveolar regions of the lung are biodegraded (shortened) prior to rapid clearance (Warheit et al., 1992). In addition, in a 4-wk inhalation study in rats with Nylon RFP (mean length, 9.8 μm; mean diameter, 1.6 μm), lung clearance of Nylon RFP was rapid, beginning 1 mo postexposure and through the 12-mo postexposure period (Warheit et al., 2003).

5.6. Chemical Composition

Surface Reactivity. Surface composition regulates fiber uptake, protein adsorption, free radical generation, and release of metallic ions, which are processes implied in the pathogenic mechanisms elicited (reviewed in Fubini et al., 1998). Most published studies have dealt with crystalline fibers such as asbestos, and fewer studies have dealt with the activity of amorphous fibers.

The presence of iron at the fiber surface plays a crucial role in most of the above processes (Hardy & Aust, 1995; Keeling et al., 1994; Kamp & Weitzman, 1999). Iron may be an integral constituent in the chemical composition of the fibers, as in most amphibole asbestos and in slag and rock wools, may be present as substitute of similar ions (Mg^{2+} in chrysotile asbestos), or may be an impurity acquired from the environment or endogenously (reviewed in Fubini & Otero-Arean, 1999).

Free Radical Generation. Mineral fibers may generate free radicals and reactive oxygen species (ROS) in cell-free models and ROS and reactive nitrogen species (RNS) in vitro cell cultures. Weitzman and Graceffa (1984) were the first to report a consistent release of ·OH from various asbestos types in cell free systems. Fiber-generated and cell-generated reactive species may subsequently react; for example, the radical species $O_2^{\cdot-}$ and NO, yielding peroxynitrite (OONO· -) or $O_2^{\cdot-}$ and also free oxygen and H_2O_2 yielding the hydroxyl radical (·OH) in the presence of transition metal ions (Halliwell & Gutteridge, 1986; Fubini et al., 1998). This free radical is a highly reactive species, capable of causing, among other deleterious effects, DNA damage, protein oxidation, and lipid peroxidation (Hardy & Aust, 1995; Kamp & Weitzman, 1999; Aust & Eveleigh, 1999).

At least three different mechanisms of surface-generated free radicals may take place, each one triggered by a different type of active surface site (see Box 1):

1. Fenton chemistry (Fe^{2+} in presence of H_2O_2 yields ·OH).
2. Haber–Weiss cycle (in the absence of H_2O_2 and Fe^{2+}, endogenous reductants allow progressive reduction of atmospheric oxygen to ·OH).
3. Homolytic rupture of a carbon–hydrogen bond in biomolecules, with formation of a carbon-centered radical.

While mechanism 1 is relevant only in biological compartments where H_2O_2 is present, mechanisms 2 and 3 may occur ubiquitously. Traces of iron are sufficient to trigger mechanism 1 on most mineral fibers, and radical yield is unrelated to the amount of surface iron (Fubini et al., 1995; Fubini & Mollo, 1995). Chrysotile and some mineral fibers with low iron content exhibit, in fact, a radical-generating potential close to that of the amphiboles. Mechanism 2 is active in most asbestos forms, provided ferrous iron is present (Pézerat et al., 1989; Hardy & Aust, 1995; Fubini et al., 1995; Fubini & Mollo, 1995; Gulumian et al., 1999) and in some cases correlates with DNA base hydroxylation (Nejjari et al., 1993). The surface sites involved in this reaction, however—few isolated and poorly coordinated iron ions—become inactive following surface modifications brought about by thermal treatments (Fenoglio et al., 2001; Tomatis et al., 2002a) or chelating agents (Martra et al., 2003). Mechanism 3 either may be directly triggered by surface sites or may be the effect of the reaction of a short-lived radical, for example, ·OH, with the target molecule. For all mechanisms there is now considerable evidence that not all iron species are equally bioactive (Gulumian et al., 1993a, 1993b, 1999; Fenoglio et al., 2001; Martra et al., 2003), while powdered iron oxides are fully inactive (Costa et al., 1989; Fubini et al., 1995; Fubini & Mollo, 1995). Amphibole asbestos samples heated up to 400°C in air (Tomatis et al., 2002a) or selectively deprived of ferrous iron (Fubini et al., 1995) lose their potential for mechanisms 2 and 3, but retain reactivity for mechanism 1, as long as their crystal structure is preserved (Tomatis et al., 2002a).

1) $Fe(II)_{(surface)} + H_2O_2 \rightarrow Fe(III) + OH^- + \cdot OH$
2) Reductant + $Fe(III)_{(surface)} \rightarrow$ oxidized reductant + Fe (II)
   $Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\cdot-}$
   $O_2^{\cdot-} + 2H^+ + e^- \rightarrow H_2O_2$
   $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + \cdot OH$
3) $-C-H + X_{(surface\ site\ or\ free\ radical)} \rightarrow -C^{\cdot} + HX$

BOX. 1. Mechanisms of fiber-generated free radicals.

Iron Removal or Deposition. Iron may be removed in substantial amounts from asbestos by endogenous chelators. DNA single-strand breaks (SSBs) were related to the extent of iron removed from the fibers (reviewed by Hardy & Aust, 1995). Induction of DNA SSBs decreases in crocidolite deprived of iron by desferrioxamine (Chao & Aust, 1995). Iron depleted crocidolite undergoes a progressive amorphization of the external layers (Mollo et al., 1994) and disruption of its crystal structure (Prandi et al., 2002), suggesting strong chelators as possible decontaminating agents.

Comparing various natural and artificial fibers (amosite and crocidolite asbestos, refractory ceramic fibers [RCFs], SVFs), there was a poor correlation of the ability to cause oxidative DNA damage in vitro with iron release (Gilmour et al., 1995). Moreover iron-enriched crocidolite did not increase SSBs in DNA, but more iron was removable by chelators (Hardy & Aust, 1995b). DNA damage appears thus more related to the potential of fibers to generate free radicals than to their mobilizable or bulk iron content. On variously heated amosite and crocidolite, Fenton chemistry (mechanism 1) correlates with supercoiled DNA damage better than mechanisms 2 and 3 (Otero Aréan et al., 2001, Tomatis et al., 2002a).

Deposition of iron causes conflicting effects, likely related to the thickness and the chemical nature of the deposited layer. Iron deposition increased the ability to induce DNA damage in iron-deprived crocidolite (Hardy & Aust, 1995b) and erionite (Eborn & Aust, 1995), and in larger amounts caused "detoxification" of crocidolite (Gulumian et al., 1993b) and reduced cytotoxicity and morphological transformation of embryo cells by ceramic fibers (Elias et al., 2002). Iron supplementation inhibited crocidolite stimulation of nitric oxide synthase (NOS) activity and

expression in glial and alveolar murine macrophages (Aldieri et al., 2001).

Ferritin, a component of ferruginous bodies (Churg & Warnock, 1981), is strongly adsorbed on amosite and crocidolite asbestos (Fubini et al., 1997). In agreement with the increased DNA damage found for amosite-core asbestos bodies when compared to the effect produced by the naked fiber (Lund et al., 1994), ferritin-covered amosite caused, with traces of ascorbic acid, enhanced radical damage to DNA (Otero Areàn et al., 1999). In contrast, iron-enriched crocidolite fibers ex vivo showed a decrement in oxidant generation (Ghio et al., 1997).

Fiber Coating and Protein Adsorption. The bioactivity of an inhaled fiber may be influenced by the adsorption of proteins and lipids from the fluid lining the respiratory tract, and in vitro the following effects are seen. Immunoglobulin G enhanced the macrophage response to chrysotile but not to crocidolite, because of the opposite surface charge of the two fiber types (Scheule & Holian, 1990). Vitronectin, but not fibronectin or other proteins, increased crocidolite fibers internalization by mesothelial cells (Boylan et al., 1995). Opsonization with immunoglobulin enhanced stimulation of macrophages by long but not by short amosite fibers (Hill et al., 1995). In spite of the higher surface area of short fibers relative to the long ones, the long ones adsorbed threefold more immunoglobulin than the short ones. Incubation of chrysotile asbestos in dipalmitoylphosphatidylcholine (DPPC, a major component of pulmonary surfactant) suppressed membranolysis, but genotoxic effects were either unaffected or only partially decreased, depending on fiber length (Lu et al., 1994). In another study (Brown et al., 1998a), when crocidolite asbestos or a range of SVFs was coated with immunoglobulin G (IgG) or lung surfactant, there were distinct fiber-specific effects. Coating with IgG enhanced the ability of asbestos, silicon carbide, refractory ceramic, and glass wool fibers to stimulate an oxidative burst but had no effect on heated refractory ceramic fibers or special-purpose glass fibers in this regard. In contrast, coating with surfactant inhibited the ability of all fibers to stimulate an oxidative burst.

Surface Hydrophilicity/Hydrophobicity. The degree of surface hydrophilicity/hydrophobicity regulates cell surface adhesion, protein denaturation, and uptake of endogenous molecules (Fubini et al., 1998). Heating silica-based materials progressively converts hydrophilic surfaces into hydrophobic ones. Heated ceramic fibers showed less affinity for the surface of V79-4 cells and a lower toxicity toward these cells and toward macrophage-like cells (Brown et al., 1992). RCF3 (from the TIMA bank) heated for 24 h at 800°C became fully hydrophobic and much less cytotoxic and transforming than the original ones (Tomatis et al., 2002b)

Chemical Composition and Pathogenicity. Chemical composition affects both surface reactivity and biopersistence and thus is a determinant of fiber pathogenicity. Studies with silica-based vitreous artificial fibers (SVFs) tend to suggest that all of the variability in the experimental pathogenicity of respirable fibers can be explained by three factors: dose, biopersistence, and length. However, in many cases composition clearly plays a more complex role. The combinations of several surface properties (e.g., reactive transition metals, hydrophilicity, reactivity toward protein and peptides), in fact, and not one single feature, determine pathogenicity for many fibers (Fubini et al., 1998; Fubini & Otero Arean, 1999). For example, erionite, a fibrous form of zeolite, was almost 100% effective in producing mesotheliomas in the rat by inhalation at high concentrations (Wagner et al., 1985) yet was not especially long (about 2% longer than 20 μm). Davis et al. (1996) also reported on a sample of silicon carbide fibers that was exceptionally active in causing mesotheliomas following inhalation exposure at high concentrations, but again was not especially long (about 10% longer than 20 μm). Silicon carbide fibers showed a very marked ability to stimulate the release of tumor necrosis factor (TNF)-α from macrophages (Fisher et al., 2000) but did not appear to operate through oxidative stress mechanisms (Brown et al., 1998b) and were low in bioavailable iron (Fisher et al., 1998). Erionite may be able to accumulate biological iron in reactive form (Eborn & Ault, 1995). While new experimental data are required to correlate each surface property to a given biological response, there is currently no single physicochemical parameter that can be used to predict carcinogenicity for all fiber classes. Surface features reported to play a role in the overall biological outcome also should be considered.

6. TESTING METHODS

Conducting chronic inhalation studies in rodents for every new fiber introduced into commerce is not practical, considering the large number of fibers that are continually being developed and proposed for use. These studies are very costly (>$4 million for a single fiber), typically use hundreds of animals, take more than 3 yr to accomplish, require sophisticated exposure facilities, and are technically difficult. *Therefore, it is incumbent on the scientific community to identify shorter, simpler assays that are adequately predictive.*

Short-term assays are defined here as those that are 3 mo or less and should be aimed at both cancer and noncancer endpoints. Based on an understanding of the mechanisms of lung disease caused by fibers, it may be possible to define a tiered system that allows investment in the testing process to be minimized by allocating fibers to predicted low, medium, or high hazard categories, at any stage. Several such tiered strategies have been suggested previously (Meldrum, 2002; Fubini et al., 1998; Vu et al., 1996; Brochard & Bignon, 1995; McClellan et al., 1992). In deriving a tiered strategy, it is important to recognize the limitations of the available assays. Many of the tests are unvalidated and have been used with only a few fibers of limited specific type, have not been validated against animal or human study results, and have not been studied in mixed dust exposures, such as invariably occur in workplaces.

The report of the 1995 U.S. EPA workshop on testing of fibers (Vu et al., 1996) stated: "In this workshop the expert panel concluded that at present no single assay or battery of short-term

assays can predict the outcome of a chronic inhalation bioassay with respect to carcinogenic effects" (p. 211). The situation is somewhat changed 9 yr later, at the time of writing of the present document, due to the accumulation of data demonstrating the importance of biopersistence. The Vu et al. report identified three tiers of testing:

Tier 1. Evaluation of the physicochemical properties of the fibers.
Tier 2. In vitro tests.
Tier 3. Short-term in vivo tests.

6.1. Deriving a Sample for Testing

The starting point in deriving a sample for testing in such a tiered approach (Figure 4) is the current dominant hypothesis for the mechanism of fiber-mediated lung injury and disease, namely, that it is the long, respirable, biopersistent fibers that are the most pathogenic. It is important to point out that the tests envisaged are tests for hazard identification. There will not necessarily be data on exposure assessment, and the approach can be seen as a "worst-case scenario"—that is, the testing of samples enriched for long fibers at high exposures, when the human experience may be characterized by very much lower exposures.

Based on what is known from the silica-based synthetic vitreous fiber paradigm, the initial step should involve the preparation of a sample of long, respirable fibers (if there is potential for exposure to such fibers during the life cycle of the product) in order to avoid false negatives from the use of samples that are short. In the RCC studies (Bernstein et al., 2001a, 2001b) the starting point was fibers that had a mean length of 20 μm and that had a diameter less than 3 μm. If short fibers were to be used in the screening test, then this could produce a false negative since length is known to be an important factor in dictating pathogenicity.

The U.S. EPA guideline for chronic testing of respirable fibrous particles recommends the following:

> To maximize sensitivity of animal inhalation exposure studies to health effects of fibers, the test material should consist of rat-respirable fibers which should be enriched with the most potent fraction of long, thin fibers or fibers with high aspect ratios. As far as is technically feasible, the aerosol should be cleaned up from non-fibrous particles. The aerosol should be characterized in terms of fiber and non-fiber/particle size and number; fiber number should be expressed by total fibers and by fiber length, e.g., WHO fibers (greater than 5 μm in length), fibers greater than 10, 15 and 20 μm in length. If enriching the test aerosol with long, thin fibers is not feasible, the reasons should be clearly stated and justified, and the enrichment should be for the longest fibers or fibers with the highest aspect ratios available. The aerosolized fibers should be discharged to Boltzmann equilibrium before being delivered to the test species. (U.S. EPA, 2001, p. 4)

For the investigation of the biopersistence of fibers it is essential that the diameter also is clearly defined. The European Union (EU) recommends that fibers used in biopersistence tests should have a geometric mean diameter as close to 0.8 μm as possible and longer than 20 μm if technically feasible (EU, 1997). The geometric mean diameter of those fibers longer than 5 μm should be as close to 0.6 μm as possible, if technically feasible. In addition, for studies involving the evaluation of any pathological endpoints, the number and size distribution of fibers should be similar. Of particular importance as well is that the number of nonfibrous particles should also be comparable between fiber samples. For the aerodynamic sizing of the fibers, bulk materials without binder should be used because the binder material can lead to an enrichment of the granular binder particles.

Therefore the starting point should be a system for deriving a sample of fibers with the dimensions described above if they are relevant to the exposure scenario. A long respirable fiber sample should then be used in the tests for biological activity. Since dissolution in vitro is not based on assessing long fibers, it does not necessarily require preparation of a long fiber sample.

Because the average diameter of most bulk fiber materials is greater than 10 μm, extensive size separation procedures are necessary to isolate the long respirable fiber fraction. For example, it typically requires processing of 1000 kg of bulk fiber to obtain 10 kg of fiber with average dimensions of 1 μm $\times$ 20 μm. It should also be borne in mind that short fibers and granular fragments could make a contribution to adverse noncancer effects (Bellmann et al., 2001), so nonfibrous particles should be kept to a minimum in the test sample. There may be a case for enriching for long fibers but ensuring that there is a short fiber component, as this more closely mimics the workplace exposure and the short fibers are likely to impact on a different compartment of the lung (overload/clearance/toxicity) from that of the long fibers.

Preparation of synthetic organic fiber (SOF) samples for toxicity testing has its own unique challenges. Because the diameters of most organic fiber types are too large to be rat respirable, significant efforts are often necessary to derive an appropriate sample of rat-respirable fibers for testing. Moreover, a new nomenclature has been established for respirable SOFs (referred to as RFP, respirable-sized fiber-shaped particulates) to distinguish them from the nonrespirable forms. Three examples demonstrate the complexity of generating samples for testing:

1. In order to conduct a study with *p*-aramid RFP, a sample of *p*-aramid pulp (containing <40% respirable fibers) was extensively processed in a pulping operation for several weeks.
2. Similarly, to conduct a 4-wk inhalation study with Nylon RFP in rats, the test substance, supplied as white, 18-denier, trilobal chopped and ground fibers, was washed (to remove fiber-finishes), dried, and cut in a flocking plant to (a) simulate exposures to workers in the flocking industry and (b) generate respirable samples of fibrous Nylon. It should be noted that in order to conduct a 4-wk inhalation study with respirable Nylon RFP aerosols, a small fraction, only <10% of the original starting material (1000 lb), ultimately was used for the inhalation studies, requiring more than a

year of technical effort for Nylon processing. Moreover, the ratio of WHO fibers to nonfibrous particulate in the sample was 1:10–20. Although this was an unintended consequence of the fiber preparation, this ratio better simulated the occupational environment in the flocking plants.

3. Recently, a new organic fiber type was supplied for a preliminary pulmonary bioassay toxicity screen. This nonrespirable SOF type (which was supplied on a spool) has never been utilized commercially; thus, the nature of future occupational exposures is unclear. After initial processing in a pulping operation, the fiber preparation has been subjected to a variety of additional processing strategies in order to obtain a "respirable sample" that can be utilized in an intratracheal instillation pulmonary bioassay study.

To summarize, the silica-based vitreous fiber paradigm for preparing a respirable fiber sample may not be relevant for processing and preparing synthetic organic fibers for toxicology tests (Warheit et al., 1992, 2001a, 2002).

6.2. Benchmark or Control Fibers

It is desirable that benchmark fiber samples be utilized in these tests wherever possible to serve as positive and negative controls. It may not be necessary for a given institution to include positive and negative controls in every study, as this would add considerably to cost, and recent historical data may suffice. A rational benchmark fiber panel would consist of a biopersistent fiber (vitreous or long amphibole) and a biosoluble fiber (e.g., MMVF34); these should be prepared according to the same guidelines for length and respirability and should be tested in parallel with the test samples. The dimensions (length and diameter bivariate distribution) of the control fibers should be as close as possible to the dimensions of the fibers to be tested; deposited lung burdens should match those of the test fibers in terms of fiber sizes and fiber number concentrations. This may require adjustment of the inhaled concentration if the aerodynamic properties of the control fibers and of the test fiber differ significantly (e.g., difference in specific density). The amount of nonfibrous particles should be similar in control and test fibers.

A further example of benchmarking may occur when newer fiber types are used that may cause novel types of adverse effects. It is, for example, possible to envisage an organic fiber causing immunopathological effects. In this case the adverse effect should be benchmarked and a relevant control utilised that produces the same adverse effect. For example, if extrinsic allergic alveolitis were suspected to be caused by an organic fiber, then a suitable control inhaled allergen (e.g., ovalbumin) in a suitable regimen should be utilized.

[At the present time, no benchmark organic fiber types are available to serve as controls for organic fiber studies.]

6.3. Physicochemical Characterization

Any new fiber type should be characterized before in vitro and in vivo testing. The following recommendation for testing strategy is partly drawn from the ECVAM Workshop Report Number 30 (Fubini et al., 1998).

Characterization of Fibers as Received (Bulk)

Where applicable, fiber samples for in vitro and instillation studies and starting material for inhalation studies should be examined to evaluate the following features:

- Chemical composition of the bulk (inductively coupled plasma–mass spectroscopy, ICP-MS).
- Crystallinity (x-ray diffractometry [XRD] or transmission electron microscopy [TEM]).
- Specific surface (BET method).
- Morphology of fibers (TEM/SEM).
- Chemical composition of outmost layers (SEM/TEM + EDS [electron dispersion analysis]).

Wetability and the presence of binders should also be assessed:

Care should be taken that before any test the fibers are:

- Washed and stored in a clean environment.
- Mildly sonicated to avoid agglomeration (high-energy sonication may activate chemical bonds).
- With cell culture tests, the cell growth medium should not contain quenchers of radicals or ion chelators.

Characterization of Fibers in Aerosol for Inhalation Studies

For inhalation studies of biopersistence or for short-term studies of biological effects, the following properties should also be defined:

- Morphology of airborne fibers (TEM/SEM).
- Airborne mass and fiber number concentration.
- Nonfibrous particle number and dimensions.
- Mass median aerodynamic diameter and geometric standard deviation (GSD).
- Size distribution of length and diameter of the airborne fibers, including the number of WHO fibers per unit volume and the proportion of fibers $>20\ \mu$m per unit volume.

Characterization of Fibers Recovered From the Lungs

Where applicable, the assessment of fibers recovered from the lungs should involve a lung digestion procedure (e.g., hypochlorite), validated for the fiber in question, which does not attack the fiber surface or change the dimensions, followed by quantification of the following aspects of the lung burden:

- Content and characterization of fibers and particulates per unit lung mass.
- Morphology of the recovered fibers (TEM/SEM, if necessary).
- Fiber mass.
- WHO fiber number and number of fibers $>20\ \mu$m long.

- Fiber diameter and length (bivariate size distribution).
- Chemical composition of the bulk (ICP-MS).
- Chemical composition of outmost layers (SEM/TEM + EDS).

6.4. Durability and Biopersistence

Durability or biopersistence of fiber samples has been estimated using composition data, in vitro methods, and in vivo methods.

Durability Evaluated on the Basis of Composition

Models have been proposed that provide a correlation between fiber chemical composition and acellular in vitro solubility over well-defined ranges of chemical composition expressed as oxide mass percent. For glass wool compositions the predictions are based on a linear fit of the log of the pH 7.4 in vitro dissolution rate (Eastes et al., 2000c). For stone (rock) wool compositions the predictions are based on a linear fit of the log of the dissolution rate calculated from in vivo fiber clearance data (Eastes et al., 2000a, 2000b). There are two sets of equations for stone wools, one for the low-alumina and one for the high-alumina compositions. Figure 5 is a graph of measured in vitro dissolution rates plotted against dissolution rates calculated using these equations. It can be seen that there is good correlation over a wide (>100-fold) range of compositions.

In general, these models have not been considered robust enough to be used for regulatory classification of fibers and are used only as a first line of internal screening by the fiber industry in developing new fiber compositions.

As discussed later in this article, in the European Commission fiber legislation (Council of the European Union, 1997) fibers are differentiated between initial classification into Category 2 or Category 3 based on the sum of the weight of oxides of sodium, potassium, calcium, magnesium, and barium (Sum > 18% = Category 3 and Sum < 18% = Category 2); however, chemical composition alone was not considered sufficient for exoneration of fibers from classification as a carcinogen.

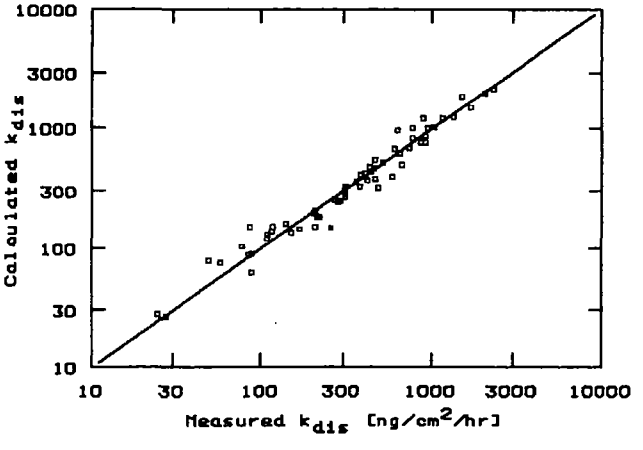


FIG. 5. Calculated versus measured dissolution rates for synthetic vitreous fibers (SVFs).

Durability In Vitro

Cell-Free Systems. It is possible to estimate the ability of fibers to persist in the lung by use of a milieu that mimics some pertinent aspects of tissue fluid. In such an environment fibers may be either completely dissolved or weakened by partial leaching of components so that they break mechanically. To determine durability in vitro, fibers are retained in an aqueous solution that has an electrolyte balance and pH close to that of physiological fluid since such factors can affect the rates of dissolution and leaching (Mattson, 1995). The pH in which fibers exist in the lungs is likely to be either pH 7.4 in lung lining fluid or as low as pH 4.5 in phagolysosomes. The solution, commonly a modification of Gamble's fluid at pH 7.4 or 4.5, has the electrolytic balance of tissue fluid although it does not contain the hallmark macromolecules of biological systems. Over time the fibers remain in a dynamic flow of this fluid or statically in fixed volume (Touray & Ballif, 1994). A number of methods have been used to follow the dissolution of the fibers; those developed through the early 1990s have been summarized in a review article (de Meringo et al., 1994). In the most widely used method, the dissolution fluid is regularly sampled and analyzed for various elements by a sensitive technique such as inductively coupled plasma–mass spectrometry (ICP-MS). From the initial fiber diameter distribution of the sample, it is possible to express the dissolution rate (K_{dis}) of any element in terms of mass lost per unit surface area per unit time—typically nanograms per square centimeter per hour. Silicon is used often used as an index element since the structural matrix of many natural and man-made vitreous fibers is siliceous. Several interlaboratory comparisons were conducted to develop and verify a standard flow-through procedure based on solution analysis. These have shown that the method is well suited for ranking different fibers with respect to dissolution rate within one laboratory but that, due to a relatively high interlaboratory variation, caution should be exercised when comparing values obtained by different laboratories (Zoitos et al., 1997; Guldberg et al., 2003; Guldberg et al., 2003).

It is also possible to follow the dissolution rate by direct measurement of mass loss. More recently, a technique has been developed to follow fiber dissolution directly by means of fiber diameter measurement (Potter, 2000). In this technique individual fibers are exposed to a continual flow of the modified Gamble's solution in a cell that allows periodic optical measurement of the diameters of the fibers while they remain immersed in the fluid. This technique has the advantage of measuring directly the parameter of interest, that is, fiber diameter, in a way that is conceptually and experimentally simple. In addition, the dissolution rate calculation does not depend on fiber composition, diameter distribution, or surface area. However, this technique cannot be used for fibers in the submicroscopic

range, for example, <0.25 μm. Several studies have shown a good correlation between the results of in vitro, cell-free dissolution measurements and fiber durability in the lung (Maxim et al., 1999; Searl et al., 1999; Oberdorster, 1996).

Cellular Systems. Fibers can be added to cells in culture and then assessed for changes in their dimensions or elemental composition over time (Jaurand, 1994). Since the exact cellular compartment in which fibers are retained during long-term residence is unknown, this approach is difficult to validate but macrophages seem a likely candidate and these cells have been used in this type of assay (Luoto et al., 1994). The difficulty with cellular systems is that they are usually static systems with very small volumes of fluid in which the potential for dissolution approaches zero, in contrast to the very high dissolution potential available in the in vivo lung. Thus, fibers in these systems are primarily differentiated based on shape rather than also on solubility.

The dissolution behaviors of various synthetic vitreous fibers were studied in serially refreshed static cultures near pH 7 with and without rat alveolar macrophages (Luoto et al., 1994, 1995). Analysis of the solutions for Si, Fe, and Al showed significant differences in dissolution attributable to the presence of macrophages in the culture. The media with macrophages typically had lower Si and higher Fe and Al concentrations than did the cell-free media. This is consistent with the presence in vivo of an additional dissolution environment that is acidic—presumably relating to the action of phagocytic cells. Experiments similar to what was just described were done but in a recirculating flow-through system (Luoto et al., 1998). The results generally show a smaller difference between dissolution with and without the presence of the phagocytic cells.

Another major problem in the in vitro systems is that the fibers are not in the lung making contact with cells and various lung compartments where normal physiological processes can affect the biopersistence of fibers. If fibers are deposited in the lungs of rats, their biopersistence can be determined by characterizing the lung fiber burden over time, and this can be assumed to give a better indication of how the fibers would persist in the lungs of exposed humans than the in vitro method.

Recent in vitro cellular studies have been reported that are designed to elucidate the mechanisms of biodegradability of organic *p*-aramid fibrils. Using an in vitro acellular system, Warheit et al. (2001b) reported that components of lung fluids coat and catalyze the *p*-aramid, thereby predisposing the respirable fibers to enzymatic cleavage. It was suggested that this mechanism could play a significant role in facilitating the shortening of inhaled *p*-aramid RFP that has been reported in the lungs of exposed rats and hamsters. In addition, in vitro cellular systems, involving alveolar macrophages and macrophage-lung epithelial cell cocultures, have been utilized to demonstrate biodegradability of *p*-aramid respirable fibers following phagocytosis of fibers by macrophages.

Biopersistence

Measurement of biopersistence of fibers in the lung is one of the most important short-term assays for estimating the potential hazard of fiber types (see section 5.5). Two different methods of exposure have been used for biopersistence studies: inhalation and intratracheal instillation (IT). Because of the limitations of IT, as discussed later, inhalation is the preferred method of exposure for biopersistence studies. Naturally, the inhalation exposure more closely mimics normal human exposure, and there is no bolus effect as seen with instillation. With either method of administering the fiber sample, the approach for determining biopersistence involves sequential sacrifices, lung digestion and characterization of the lung fiber burden, and calculation of a retention $T_{1/2}$. By using sequential sacrifices, the change in the structure, size distribution, and morphology of the lung fibers over time can be followed to provide an indication of the biopersistence (Muhle et al., 1994; Hesterberg et al., 1996; Bernstein et al., 1997).

Biopersistence Following Inhalation Exposure. In developing the European Commission fiber legislation, the relationship of the biopersistence following short term inhalation and intratracheal instillation to the results of chronic inhalation and intraperitoneal injection studies was investigated in detail using the original data from these studies. The biopersistence of fibers longer than 20 μm was found to be an excellent predictor both of the collagen deposition in the bronchoalveolar junctions (an early precursor of interstitial fibrosis) in the chronic inhalation studies and of tumor response in the chronic intraperitoneal injection studies (Bernstein et al., 2001a, 2001b). Based on this analysis, the European Commission established its fiber directive (European Commission, 1997). In Note Q of the EC Directive 97/69/EEC, it is stated that the classification as a carcinogen need not apply if it can be shown that the substance fulfills one of the following conditions:

1. A short-term biopersistence test by inhalation has shown that the fibers longer than 20 μm have a weighted half-time less than 10 days.
2. A short-term biopersistence test by intratracheal instillation has shown that the fibers longer than 20 μm have a weighted half-time less than 40 days.
3. An appropriate intraperitoneal test has shown no evidence of excess carcinogenicity.
4. Absence of relevant pathogenicity or neoplastic changes in a suitable long-term inhalation test.

A 1-wk (6 h/day for 5 days) inhalation exposure followed by 26-wk follow-up of lung burden fibers has been proposed (European Commission, 1999a), but it is notable that for more soluble fibers the trend for biopersistence is often evident at 13 wk, so a 3-mo study could be envisaged that would allow determination of biopersistence.

However, it has also been reported that prolongation of the exposure duration can lead to an increase in the observed retention

half-time of fibers. For example, the half-time in rat lungs of rock wool fiber MMVF34 fibers (length >20 μm) was 5 days after a 5-day inhalation exposure, whereas the half-time for this fiber type was 27 days after a 12-mo exposure period (Kamstrup et al., 1998). Therefore, in making comparisons of biopersistence studies, the same exposure times and mode of delivery should be used.

Biopersistence Following Intratracheal Instillation Exposure. This technique delivers the fibers as a bolus suspension into the lung via the trachea. Problems associated with this method include that the fibers are necessarily delivered in suspension form in saline or water and so some dissolution may have already occurred. The delivery of the mass burden into the lung as a suspension can also result in fiber clumping or bundles, with some fibers orientating themselves in non-aerodynamic ways across the airways. The resultant bridging of fibers across small airspaces can block deposition deeper into the lung and lead to granuloma formation and fibrosis that obstructs the bronchioles. The bolus dose also means that the fibers are in an inflammatory milieu immediately. However, fibers may be instilled at very low dose or in divided doses, and this may mitigate the problem (Driscoll et al., 2000). From the data, a retention half-time can be calculated that is longer for biopersistent fibers than for nonbiopersistent fibers, and although there is disagreement on the absolute retention half-time relationship between instillation and inhalation (Muhle & Bellmann, 1995), they rank different fiber samples in the same order. Thus, the method and duration of exposure of animals can influence the retention half-time.

6.5. Short-Term Biological Tests

There is a paradox in trying to ascertain the activity of fiber samples in very short-term assays of biological activity. There are studies that demonstrate that long, nonbiopersistent fibers that are not pathogenic in vivo are positive in tests of ability to cause proinflammatory (Ye et al., 1999) and genotoxic (Hesterberg et al., 1983) effects in short-term in vitro tests. This arises because biopersistence of fibers is known to be central to their overall pathogenicity yet very short-term assays cannot allow for this important property; thus nonbiopersistent fibers are likely to produce false positives. However, in view of the technical sophistication required and the expense of inhalation assays, these very short-term assays continue to be used.

Donaldson and Brown (1993) and Warheit (1993) each have proposed using bronchoalveolar lavage assessments and short-term pulmonary bioassays to study the pulmonary cellular responses following exposures to fibrous or particulate materials. It is generally regarded that using a short-term assay to assess biomarkers of lung injury and inflammation can provide valuable information on the nature of fiber toxicity and the potential for the development of pulmonary fibrosis.

Tests In Vitro

Dose. As mentioned earlier, while in vitro cellular tests can provide specific information, as designed, due to their static nature they cannot take into effect the important influence of fiber dissolution/breakage on these events.

Fiber Reactivity (Acellular Assays). It has been suggested that the ability of fiber samples to cause oxidative stress is a factor in their proinflammatory and genotoxic effects and that transition metals, especially iron, may play an important role in causing these effects (reviewed by Hardy & Aust, 1995a; Kane et al., 1996; Kamp & Weitzman, 1999). Other surface properties (see section 5.6) are also important and may enhance or depress the pathogenic potential of a given fiber type.

Bioavailable and bioactive transition metals. The ability of asbestos fibers to activate a number of pathways signaling for inflammation and other relevant pathways for proliferation and related effects has been shown to depend on iron (Luster & Simeonova, 1998; Kamp & Weitzman, 1999). Cell-free and cellular tests (reported in section 5.6) have clearly shown that neither all the iron contained in the fiber, nor what is at the surface and may be mobilized (bioavailable), is active in this respect (bioactive). Accordingly, one study of pathogenic and nonpathogenic fibers showed no tendency for the pathogenic fibers to release more bioavailable transition metals than the nonpathogenic fibers in vitro (Fisher et al., 1998). Transition metals can take part in the various mechanisms (see Box 1, section 5.6) whereby the hydroxyl radical—which adversely affects a number of biological molecules such as protein, lipids, and DNA—is generated. Proof of a role for transition metals in a given test is normally given by the diminution of the effect following addition of the transition metal chelator deferoxamine. Therefore, bioavailable transition metal release from a fiber, associated with evidence of bioactivity, may be important in determining toxicity of any sample (Lund & Aust, 1992). In the future, fibers may be developed that contain a range of metals that are reactive in biological systems, and these can be detected by a number of sensitive methods including ICP-MS. Reactive species other than transition metals, such as organics, could be a component of future fibers, and these need to be considered.

Free radical release. Several direct and indirect methods for the detection of free radicals are available and have been used with fibers (reviewed in Fubini, 1996).

Electron paramagnetic resonance (EPR) can be used by means of the spin trapping technique to detect the nature and amounts of free radical species generated by any fiber sample in acellular tests (employed, by many authors since the pioneering study of Weitzman & Graceffa, 1984). Such technique also allows analysis of the kinetics of release, providing information on fiber types in which slow radical release may cause sustained inflammation (Fenoglio et al., 2001). When applied in vivo for asbestos, such technique showed that the greater the radical production, the more likely the fiber sample is to cause oxidative stress in the lungs (Ghio et al., 1998).

Another method is measurement of DNA damage. The ability of fibers to cause scission of plasmid DNA is a measure

of hydroxyl radical activity that has been used to demonstrate the ability of asbestos fibers to generate ROS (Gilmour et al., 1997). This technique did not discriminate between a panel of pathogenic and a panel of nonpathogenic fibers. However, ceramic fibers were very active in this assay (Brown et al., 1998b). The role of this type of assay in determining the pathogenicity of organic fibers is unknown, since the mechanism of their pathogenicity (if any) is unknown. The generation of 8-OH DG, following the incubation of fibers with naked DNA, has also been used as a test (Nejjari et al., 1993). This showed that iron-containing fibers such as crocidolite and amosite were the most reactive, whereas fibers without iron were inactive.

Also available are other assays of the ability of fibers to generate oxidative stress in cell-free systems. Various chemical/biochemical assays that detect the ability of fibers to generate free radicals are available. The salicylic acid assay (Maples & Johnson, 1992) showed a relationship of free radical reaction with the ability (or lack of ability) to cause mesotheliomas in rats and humans for a panel of fibers that included erionite, crocidolite, amosite, anthophyllite, chrysotile, JM code 100 glass fibers, and glass wool.

Depletion of antioxidants. Depletion of the antioxidant defenses may also contribute to the oxidative stress. This may be caused by the inhibition of some cellular pathways (such as the pentose phosphate pathway, via the inhibition of its rate-limiting enzyme glucose-6-phosphate dehydrogenase (Riganti et al., 2002), or by the simple consumption at the fiber surface of endogenous antioxidants. Several fiber types, including asbestos, glass wools, and ceramic fibers, were tested for their ability to deplete ascorbic acid and glutathione in lung lining fluid as a measure of the potential to cause oxidative stress in vivo (Brown et al., 2000); in this study the greatest depletion of antioxidants was observed with the two noncarcinogenic glass wools, suggesting that such reactivity cannot per se confirm toxicity, but may augment the oxidative stress caused by other factors.

Hydrophilicity/hydrophobicity. Fully hydrophobized ceramic fibers (Tomatis et al., 2002b) or crystalline silica particles (Fubini et al., 1999) lose much of their original cytotoxic and transforming potential. Amphibole asbestos heated at 800°C, although still partially hydrophilic, lost its ability to generate free radicals (Tomatis et al., 2002a) and damage DNA (Otero-Arean et al., 2001). Hence hydrophobicity is a property that, likely by regulating cellular uptake and clearance, modulates fiber pathogenicity. Hydrophilicity may be measured in various ways, with comminuted material such as fiber samples; the most appropriate is water vapor adsorption—the higher the water uptake, under defined conditions, the higher the degree of hydrophilicity (Fubini et al., 1999). With substantially hydrophilic vitreous surfaces, such as glass, slag, and rock wools, water uptake, mainly by cations, parallels solubility (Ottaviani et al., 2000).

Cellular Tests

The dose used in these studies is usually very high compared to in vivo dose, and the dosimetry issue should be fully considered in deciding the usefulness of any assay. Tests that more closely mimic the conditions of the lung, such as cocultures of epithelial cells and macrophages, may be useful (Warheit, 2001b).

Macrophages. Alveolar macrophages are the initial defense mechanism against particles and fibers that deposit in the lower respiratory tract. Phagocytic cells express several types of surface receptors that recognize exogenous particulates and microorganisms, as well as endogenous ligands. Activation of different classes of surface receptors triggers phagocytosis, the respiratory burst mechanism, migration and chemotaxis, and production of proinflammatory mediators and cytokines. In general, there is a correlation between exposure to highly toxic particulates and fibers (e.g., freshly fractured quartz, asbestos fibers) and release of oxidants and cytokines from alveolar macrophages (reviewed in Driscoll et al., 2002). Opsonization or coating of exogenous particulates with activated complement fragments, immunoglobulins, or surfactant proteins (especially SP-A, which binds to the C1q collectin receptor) enhances phagocytosis and respiratory burst activity (Donaldson et al., 1992; Palecanda & Kobzik, 2000). Crystalline silica particles and crocidolite asbestos fibers are also bound by opsonin-independent receptors, especially the class A macrophage scavenger receptor (SR-A; Palecanda et al., 1999). It has been postulated that binding of inert dusts such as titanium dioxide by these scavenger receptors does not trigger macrophage activation and release of oxidants and pro-inflammatory mediators, in contrast to binding of toxic particulates such as crystalline silica or asbestos fibers (Palecanda et al., 1999). The biochemical mechanisms and signal transduction pathways responsible for the different responses of alveolar macrophages to different types of inhaled particulates are unknown.

Macrophage death. The ability of fibers to cause macrophage toxicity has been determined by various assays such as trypan blue exclusion and lactate dehydrogenase (LDH) release. Dosimetric considerations suggest that only very low numbers of fibers would normally deposit and make contact with cells in workplace exposure situations, so toxicity tests are often carried out in vitro in order to select nontoxic doses for studies at doses that show stimulatory effects that are more likely to occur in vivo. Assessment of macrophage death as an endpoint is generally useful only to select nontoxic doses for macrophage stimulation experiments.

Macrophage stimulation. Fibers can be investigated for their ability to stimulate macrophages in various ways that lead to inflammation or bystander injury to epithelial cells:

Oxidative burst: Macrophage-derived oxidants arise from superoxide anion, the product of NADPH-oxidase following the initiation of phagocytosis. There have been many studies on

the ability of fibers to stimulate the macrophage oxidative burst (Hansen & Mossman, 1987; Schuele & Holian, 1989; Hill et al., 1995), but there appears to be little specific effect of pathogenic fiber samples without opsonin such as IgG.

Inflammatory mediators: Fibers have been shown to stimulate release of various inflammatory mediators from macrophages, including eicosanoids, cytokines, and chemokines (Leikauf et al., 1995). Oxidative stress is proposed as a central mechanism resulting in release of the mediators. Oxidative stress-activated genes are controlled by the transcription factor nuclear factor (NF)-κB. Under the action of oxidative stress, NF-κB separates from its inhibitor and migrates to the nucleus, where it can bind to the κB consensus sequence in the promoter region of many pro-inflammatory genes such as TNFα, ICAM-1, and COX2, causing them to be expressed. Studies on the ability of fibers to activate NF-κB have been described for a number of fibers (Schins & Donaldson, 2000) using the electrophoretic gel mobility shift assay (EMSA) (Cheng et al., 1999) or immunocytochemistry to demonstrate the nuclear localization of component of NF-κB (Brown et al., 1999). In addition to measuring the signaling pathways for the expression of pro-inflammatory proteins, the proteins themselves should be measured (e.g., IL-8, TNFα, IL-1b, etc.) (Fisher et al., 2000; Luster & Simeonova, 1998; Simeonova et al., 1997). Constitutive NF-κB activation has also been proposed to play a role in carcinogenesis (Karin et al., 2002).

Pentose phosphate pathway: There has been a recent report that crocidolite asbestos, but not glass wool (MMVF10), inhibits the pentose phosphate pathway (PPP) in epithelial cells. The PPP generates NADPH, so inhibition of the pathway results in NADPH "debt." NADPH is not, therefore, available to act as a cofactor for various antioxidants, so antioxidant defense is impaired, leading to oxidative stress (Riganti et al., 2002).

Genotoxicity Tests

Epithelial Cells Epithelial cells are involved in proinflammatory effects and are also the cell of origin for bronchogenic carcinoma, so can form the basis of testing for both of these endpoints.

Genotoxic Effects. The ability of fibers to cause genotoxic effects in vitro has been demonstrated. Promoting effects of fibers include the ability to stimulate proliferation, so effects on genes associated with the regulation of the cell cycle, such as c-fos and c-jun, are of interest, as well as direct measurement of effects on cell proliferation. There are numerous genotoxic endpoints that can be utilized-including:

- Micronucleus formation (Kodama et al., 1993).
- Sister chromatid exchanges (Kodama et al., 1993).
- DNA damage (Comet assay) (Puhakka et al., 2002).
- Deletions (Hei et al., 2000).
- 8-OH-dG (Leanderson et al., 1988).

Mesothelial Cells, Epithelial Cells, and Fibroblasts

Since mesothelial cells are the cells that undergo transformation to produce mesotheliomas, there has been considerable interest in the direct effects of fibers on these cells. It should be borne in mind that the short-term assay paradox is especially relevant for this cell type because there is a requirement for translocation of fiber from the site of deposition on the epithelial surface to the sensitive mesothelial tissue. This translocation is likely to require time, so there is even more opportunity for the influence of the lung milieu to act on nonbiopersistent long fibers to render them shorter.

A range of cellular endpoints have been measured, including the obvious ones associated with signaling for proliferation and oncogene activation (Heintz et al., 1993). Genotoxic endpoints are also highly relevant for these target cells and include all of those listed above (Fung et al., 1997).

Subchronic Biological Tests In Vivo

Subchronic inhalation studies (90 days in rats) are considered short-term assays here, and are a useful bridge between 5-day and chronic exposure studies. While the carcinogenic endpoint would not be expected in such a subchronic assay, there are a number of endpoints that can be used to position fibers as to their likelihood of causing fibrosis and/or cancer. Many changes are considered to be precancerous, including chronic inflammation, hyperplasia, metaplasia, and mutation. Fibrosis as a result of the inhalation of carcinogenic fibers can be detected within 3 mo so could be detected in a short-term assay. All fibers that have caused cancer in animals via inhalation have also caused fibrosis, and at an earlier timepoint, that is, by 3 mo. However, there have been fibers that have caused fibrosis but not cancer. Therefore, in vivo studies that involve short-term exposure of rat lungs to fibers and subsequent assessment of relevant endpoints, notably fibrosis, are probably adequately conservative for predicting long-term pathology—that is, will identify fibers that have a fibrogenic or carcinogenic potential.

Two studies with organic fibers suggest that subchronic pulmonary endpoints are operative following exposures to organic fiber types (Hesterberg et al., 1992; Bellmann et al., 2000). We may also anticipate that immunological phenomena that might result from exposure to organic fibers would be manifest in this time frame, although currently there are no data on this.

Three months is long enough for there to be clear effects of biopersistence (Bernstein et al., 1996) on the long fibers, so this should be reflected in the biological response. These studies could be done in concert with the biopersistence studies, and indeed a rational approach has been suggested where there is exposure of rats to fibers, with one subgroup of rats set aside for assessment of biopersistence and another group for biological effects. This would allow biological effects to be understood in the context of the dosimetry. However, it should be noted that the retention half-time of fibers depends on the retained dose (Muhle et al., 1995, Kamstrup et al., 1998).

Some of the parameters that should be measured in a 3-mo fiber inhalation study to provide the basis for selecting exposure levels for a chronic study are noted in the U.S. EPA Guideline for Combined Chronic Toxicity/Carcinogenicity Testing of Respirable Fibrous Particles (U.S. EPA, 2001). Parameters to be evaluated include lung weight, fiber lung burden and clearance, cell proliferation, inflammatory response markers, and histopathology. The European Commission guideline for subchronic inhalation toxicity testing of synthetic mineral fibers in rats (European Commission, 1999b) specifies similar parameters.

Exposure concentrations in a subchronic fiber inhalation study should be selected to ensure that the biological effects of a pathogenic fiber would be detectable at least at the highest exposure level. The U.S. EPA Guideline indicates that the highest concentration tested in the chronic study should be set at a level at which some degree of impaired clearance and toxicity are observed. Thus, a similar requirement is implied for the subchronic study that provides the data on which chronic dose selection is based. The EC guideline for subchronic inhalation toxicity testing of fibers (European Commission, 1999b) specifies 3 exposure concentrations, the highest of which should be 150 fibers/cm^3 with length greater than 20 μm, if technically feasible.

The European Commission recently completed a "calibration" assay (Bellmann et al., 2003) in which a 3-mo inhalation study investigated the biological effects of a special-purpose glass microfiber (E-glass microfiber), the stone wool fiber MMVF21, and a new high-temperature application fiber (calcium-magnesium-silicate fiber, CMS) in Wistar rats. Rats were exposed 6 h/day, 5 days/wk, for 3 mo to fiber aerosol concentrations of approximately 15, 50, and 150 fibers/ml (fiber length >20 μm) for E-glass microfiber and MMVF21.

The biological effects measured included inflammatory and proliferative potential, histopathologic lesions, and the persistence of these effects over a recovery period of 3 mo. Generally, observed effects were higher for E-glass microfiber when compared to MMVF21. The following clear dose-dependent effects on E-glass microfiber and MMVF21 exposure were observed as main findings of the study: increases in lung weights, in measured biochemical parameters and polymorphonuclear leukocytes (PMN) in the bronchoalveolar lavage fluid (BALF), in cell proliferation (BrdU response) of terminal bronchiolar epithelium, and in interstitial fibrosis. The values observed in the proliferation assay on the carcinogenic E-glass microfiber indicate that this assay may have an important predictive value with regard to potential carcinogenicity. Results of the CMS exposure group indicate that effects may be dominated by the presence of nonfibrous particles.

Unfortunately, due to the large and varying number of particles and short fibers in the different fiber groups, alterations in these parameters could not be correlated specifically with fiber concentration, either of long fibers or WHO fibers. The authors concluded that if this protocol is to be used to differentiate fiber types, the number and bivariate length–diameter size distribution of the fibers and especially the content of particles should be similar for each fiber type.

The preferred route of exposure for the 3-mo study is by inhalation, but for a batch of experimental fibers that cannot be obtained in sufficient numbers to generate a cloud, intratracheal instillation may suffice if the fiber number is kept low (Driscoll et al., 2000). However, there is no database for the relationship of pulmonary fibrosis to long term effects with intratracheal instillation.

Dosimetry and Clearance

Characterization of lung fiber burden is a key aspect of fiber dosimetry. The lung fiber burden can be examined in a variety of ways; the ideal method will be dictated by the type of fiber being studied. Whatever method is chosen, it must be able to evaluate ("capture") all of the fibers and nonfibrous particulates that are retained in the lung without altering their composition or morphology. This may require the use of electron microscopy if the fibers are submicroscopic in size. It is particularly important to record the number of fibers by bivariate size distribution and number per milligram dry weight, or per gram wet weight, and per lung. These data are particularly important for evaluating the lungs at the end of the exposure period and during the "recovery" period.

Fiber Burden in the Pleura. A few studies have been done to evaluate the number of fibers in the pleura of rats (Gelzleichter et al., 1999) and hamsters (McConnell et al., 1999). The diaphragm can be collected and digested to determine if fibers have penetrated the pleura of the lung. The number of fibers can be measured for size and counted as the number per square millimeter of surface area. While the database is now limited, a robust one could be established over time. The main value at this time would be a yes/no answer—that is, are there fibers in the diaphragm, and what are their dimensions?

Fibers have been recovered from the pleural cavities of rats (Gelzleichter et al., 1996) and humans (Boutin et al., 1996). Although rat alveolar macrophages are phenotypically different from rat pleural or peritoneal macrophages (Gjomarkaj et al., 1999), it is not known whether pleural macrophages show the same responses to these translocated fibers as alveolar macrophages. Release of proinflammatory mediators from alveolar or pleural macrophages results in accumulation of neutrophils and newly recruited monocytes from the peripheral blood, which can further amplify the inflammatory reaction and produce tissue injury. It is proposed that long, biopersistent fibers that are not easily cleared from the lower respiratory tract have a greater potential to perpetuate these inflammatory reactions and cause tissue injury (Hesterberg et al., 1994). This is the biologic rationale for proposing persistent inflammation as an endpoint for potentially toxic fibers in short-term in vivo assays.

Impairment of Clearance. In addition to the normal characterization of the lung fiber burden, it has been suggested that conditions of particle/fiber overload and impaired clearance in

the lung can be detected by analysis of the fiber burden in the lung-associated lymph nodes (Bellmann et al., 2002). Impairment of clearance also could be assessed via challenge with a tagged particle (Vu et al., 1996). The clearance of the labeled particles should be measured over a period of a few months. By this method, in addition to retention measurements of fibers, it is possible to distinguish between fiber clearance and macrophage-mediated clearance. The latter is important in determining whether a dust overload condition may have occurred, whereas fiber clearance may be due to dissolution or breaking of fibers.

Lung Weight

Lung weight is an indirect and nonspecific measure of pulmonary response. Lung weight is easy to determine and can be a useful endpoint. However, increases in lung weight following pulmonary exposures may be due to various factors, including edema, particle overload, pulmonary fibrosis, and other factors. Moreover, with regard to fiber-related adverse pulmonary effects, significant increases in the weight of the lung of rodents may not become apparent until the pulmonary fibrosis is of some magnitude (usually 3+ mo). Therefore, this parameter may be of little value in assessing lung effects when using a short-term assay.

Inflammation

Inflammation is a common feature of fiber exposure and can be evaluated qualitatively or semiquantitatively (i.e., minimal, mild, moderate, marked), using standard histopathologic techniques.

Bronchoalveolar Lavage. Inflammation can be evaluated more quantitatively using bronchoalveolar lavage (Creutzenberg et al., 1998). One of the problems in using this endpoint for hazard evaluation is that fibers vary in their ability to stimulate an inflammatory reaction as defined by an increase in neutrophils and lymphocytes. While these types of cells are commonly found with crystalline materials, including fibers, they are not readily stimulated with amorphous fibers.

Pleural Lavage. Changes in lavageable inflammatory cells of the pleural cavity after asbestos exposure in rats have been reported (Oberdörster et al., 1983). In particular, an influx of peroxidase-positive macrophages 1 to 3 days postexposure was found, indicating newly arrived macrophages. This could possibly be induced by fibers translocating to the pleural space (Gelzleichter et al., 1999). Since pleural lavage can easily be performed, results of this method can be a valuable addition to lung lavage analysis.

Cell Proliferation

The investigation of epithelial cell proliferation in the terminal bronchioles and in the lung parenchyma after subchronic fiber inhalation has proven to be a useful assay to predict potential carcinogenicity (Bellmann et al., 2003). Proliferating cells are labeled, for example, by 5-bromo-2′-deoxyuridine (BrdU), which has to be administered a few days prior to sacrifice by a minipump. Cells are stained immunohistochemically using a specific antibody to the BrdU, which is incorporated into replicated DNA. The evaluation of the slides is done by analyzing an appropriate number of airway cells and of the proximal region of the pulmonary parenchyma. This assay was used also in a subchronic study after inhalation of *p*-aramid respirable fiber-shaped particulates (Bellmann et al., 2000), where resolution of the inflammatory response was correlated with a decline in proliferation (using BrdU pulse).

Rats exposed to long amphibole asbestos fibers may be used as positive controls to validate the method. In the study of Bellmann et al. (2003) using BrdU delivered by implanted minipump after subchronic exposure to the carcinogenic E-glass microfiber, the observed values of the proliferation assay up to 90 days after the end of exposure indicated a predictive value for a potential carcinogenicity. The values for the rock wool fiber MMVF21 were statistically increased only at 1 wk after the end of the subchronic exposure and not at 7 and 14 wk after the end of exposure. For future studies, the authors recommend conducting this assay up to 6 mo after termination of subchronic exposure. At this time point, a differentiation between transient and persistent proliferation may be distinguished more easily.

AP-1. The activator protein-1 (AP-1) transcription factor complex is an early-response gene that is induced in the lung by a variety of environmental and occupational toxicants, including cigarette smoke, asbestos fibers, crystalline silica, ultrafine particulates, and oxidants. These toxicants activate mitogen-activated protein kinase (MAPK) signal transduction pathways in lung target cell populations, leading to activation of the AP-1 transcription factor complex. AP-1 target genes mediate cellular adaptive responses to these toxicants, including induction of antioxidant defenses, surfactant proteins, markers of squamous differentiation, cell cycle regulators, growth factors and growth factor receptors, and extracellular matrix degrading enzymes (reviewed in Reddy & Mossman, 2002).

Upregulation of *fos* and *jun* and increased AP-1 DNA binding activity in response to asbestos fibers have been shown in lung epithelial and mesothelial cells in vitro and in vivo. Although the effects of fiber biopersistence have not been evaluated in these short-term assays, low-toxicity particles such as glass beads or titanium dioxide do not induce *fos* and *jun* in these assays (reviewed in Manning et al., 2002). A transgenic mouse model expressing a luciferase reporter gene responsive to transactivation by AP-1 has been used to confirm AP-1 activation in vivo in response to intratracheal instillation of asbestos fibers (Ding et al., 1999a) or freshly fractured crystalline silica (Ding et al., 1999b). Conceivably, this sensitive in vivo transgenic mouse assay could be used to determine whether persistent AP-1 activation is associated with biopersistent fibers in a subchronic study.

One member of the AP-1 transcription factor complex, *fra-1*, has been closely linked to lung cancer and mesotheliomas

in some rodent models and in humans (reviewed in Reddy & Mossman, 2002). Increased expression of *fra-1* has been shown in rat mesothelioma cells (Ramos-Nino et al., 2002) and in asbestos-induced peritoneal mesotheliomas in rats (Sandhu et al., 2000). Transgenic mice that overexpress *fra-1* may be useful to screen for potentially-carcinogenic man-made fibers.

Alternative methods of detecting proliferative events in treated cells and animals include measurement of activation of proinflammatory transcription factors and signaling pathways using immunocytochemistry (Hubbard et al., 2002). Proliferation in the pleura can also be detected using BrdU (Robledo et al., 2000).

Fibrosis

Although the causal association between asbestosis and lung cancer in humans is controversial (reviewed in Samet, 2000, and Nelson & Kelsey, 2002), in experimental animals lung cancer produced by inhalation of particulates or fibers is preceded by chronic or persistent inflammation and fibrosis. In 1990, Davis and Cowie reviewed the histopathologic association between pulmonary fibrosis and the incidence of benign and malignant lung tumors in a total of 144 rats from 10 inhalation studies with chrysotile or amosite asbestos. There was a statistically significant association between the extent of pulmonary fibrosis and lung tumors in these inhalation studies. Benign tumors or early malignant tumors that showed limited invasion were found originating from areas of interstitial fibrosis and hyperplasia or adenomatosis. These authors noted that this association is usually missed in chronic inhalation studies or in humans diagnosed with lung cancer because these tumors usually involve a large area of the lung and the site of origin cannot be readily identified (Davis & Cowie, 1990). Persistent inflammation and fibrosis are proposed as biomarkers for potentially carcinogenic fibers in short-term screening assays. The association of fibrosis with lung cancer is recognized to the point that fibrosis has been proposed as a surrogate for predicting the carcinogenic activity of fibers in animals (Greim, 2001).

Histological assessment of fibrosis using trichrome staining and the Wagner scale (McConnell et al., 1999) has been conducted on several studies. Typically, fibrosis is evaluated on a +1 to +4 basis. As an endpoint in a short-term study, it would be adequate to establish if fibrosis was or was not present, although the severity should also be recorded.

The process of fibrosis is also marked by increases in the local production of growth factor cytokines such as TGFβ and PDGF, and these have been detected early on in asbestos-exposed lung using immunocytochemistry (Liu et al., 1997).

Genotoxicity

Data showing effects on genes and DNA is powerful information regarding the potential of fibers to cause cancer. For example, genotoxicity can be detected in vivo as the hydroxylation adduct of guanosine, 8-hydroxy-deoxyguanosine (8-OH-DG) (Nehls et al., 1997). 8-OH-dG has been demonstrated by immunocytochemistry in lungs of rats exposed to quartz, a known carcinogen. The HPRT mutagenesis detection system has also been utilized to investigate particle-induced carcinogenesis, and mutations in this gene have been shown in quartz-exposed lung epithelial cells (Driscoll et al., 1997).

Immune Reactions and Lung Injury and Fibrosis

Altered immune responses are a potential mechanism for lung injury and should be considered in the evaluation of inorganic and organic fibers. The phenotype of alveolar macrophages may be altered by exposure to toxic particulates or fibers. Three distinct macrophage subpopulations have been defined by the following criteria: specific signals that induce activation, production of cytokines and chemokines, expression of surface markers, and production of reactive oxygen and nitrogen metabolites (reviewed by Mosser, 2003). Classical activated macrophages are involved in cell-mediated (Th1) immune responses in response to INF-γ and TNF-α. These activated macrophages express major histocompatibility class (MHC) II molecules and release TNF-α, IL-12, IP-10, MIP-1α, and MCP-1. Their phagocytic ability is reduced with downregulation of the surface mannose receptor; however, microbicidal activity is enhanced due to increased production of reactive oxygen and nitrogen metabolites. Alternative activated macrophages are immunosuppressive and are produced in response to IL-4 or glucocorticoids. These macrophages release IL-1 receptor antagonist and IL-10 with upregulation of surface mannose and scavenger receptors. These cells do not produce reactive oxygen or nitrogen metabolites and suppress production of activated T lymphocytes. It has been hypothesized that this macrophage subpopulation prevents excessive inflammatory responses to environmental agents. Finally, type II activated macrophages participate in Th2 humoral immune responses triggered by antibody complexes or ligation of toll-like receptors, CD40, or CD44. Type II activated macrophages express MHC class II molecules and release interleukin (IL)-10 and IL-13 in addition to TNF-α and IL-6 (Mosser, 2003). Several lung diseases are associated with type II activated macrophages and a local Th2 immune response, including a murine model of chronic silicosis (Huaux et al., 1999), idiopathic pulmonary fibrosis in humans (Hancock et al., 1998), and animal models of radiation and bleomycin-induced fibrosis (reviewed in Sime and O'Reilly, 2001). It has been proposed that toxic particulates induce apoptosis of immunosuppressive alveolar macrophages, thereby increasing the population of inducer or activated alveolar macrophages, which drives a persistent inflammatory response in the lungs (Holian et al., 1997).

IL-13 is a key cytokine involved in lung inflammation, alveolar "signaling," and fibrosis (Lee et al., 2001; Lanone et al., 2002). In an inducible transgenic mouse model, overexpression of IL-13 in the lung activated multiple chemokine pathways, leading to pulmonary inflammation (Zhu et al., 2002), induction of matrix metalloproteinases 9 and 12 (Lanone et al., 2002), and increased production and activation of TGF-β1 and increased expression of the hyaluronic acid receptor CD44 (Lee et al.,

2001). Persistent inflammation and upregulation of TGF-β1 are associated with asbestos-induced lung injury and fibrosis, in addition to other fibrotic lung disorders (reviewed in Sime & O'Reilly, 2001). It has not yet been determined whether exposure to asbestos or synthetic fibers may alter immune regulation in lung via the release of critical cytokines such as IL-13.

An important caveat in interpretation of these rodent models of lung injury and fibrosis must be noted. The balance between Th1 and Th2 immune responses in the lungs depends on the mouse strain and the chronicity of the disease process (Abbas et al., 1996; Huaux et al., 1999). In rodent models of silicosis, rats show a different pattern of inflammatory and immunologic responses than mice (Garn et al., 1997). Silicotic patients frequently show evidence of increased systemic humoral immunity (reviewed in Parks et al., 1999), similar to a murine model of chronic silicosis (Huaux et al., 1999). Since genetic differences in mouse strains influence Th1 and Th2 polarization of immune responses, genetic polymorphisms in humans may contribute to differences in susceptibility to the toxic and immunologic effects of crystalline silica (Yucesoy et al., 2002), as well as natural and synthetic fibers.

Chronic inflammatory conditions including silicosis and persistent viral infections are associated with an imbalance between Th1 and Th2 helper lymphocytes (CD4+ T cells). It has been proposed that a decreased cell-mediated immune response, characterized by Th1 lymphocytes, and an increased humoral immune response, characterized by Th2 lymphocytes, predispose to the development of cancer. Asbestos exposure stimulates production of several cyokines (IL-1β, TNFα, IL-6, IL-8) that may be involved in this phenotypic switch. Chronic inflammation associated with persistent asbestos fibers also upregulates COX-2, leading to increased prostaglandin (PG) E_2 synthesis, which stimulates production of angiogenic factors and activates matrix metalloproteinases (O'Byrne et al., 2000). Phenotypic switching in alveolar macrophage subpopulations or in CD4+ T helper lymphocytes may provide useful endpoints for screening potentially carcinogenic organic fibers. Angiogenesis and increased connective tissue turnover may provide a signaling local environment for cancer progression. This association provides a plausible mechanistic link between fibrosis and lung cancer (Samet, 2000).

Special Models

The majority of subchronic and chronic inhalation assays to evaluate the carcinogencity of natural and synthetic fibers have been carried out in rats. Both lung carcinomas and mesotheliomas have been produced in these assays; however, no analyses of molecular alterations in oncogenes or tumor suppressor genes have been done on these experimental tumors. Malignant mesotheliomas have been induced in rodents by direct intraperitoneal injection of asbestos fibers. Rat peritoneal mesotheliomas induced in this model system do not show mutations or deletions in the *p53* tumor suppressor gene, similar to human pleural malignant mesotheliomas. These rat tumors also fail to show any alterations at the *NF2* tumor suppressor gene locus, which is frequently mutated or shows loss of heterozygosity (LOH) in human pleural malignant mesotheliomas. In contrast, murine peritoneal mesotheliomas induced by direct injection of asbestos fibers show frequent deletion of the $p15^{INK4b}$ and $p16^{INK4a}$ tumor suppressor genes (reviewed in Kane, 2000).

Murine lung tumors induced by chemicals such as urethane, aflatoxin, nitrosamines, or polycyclic aromatic hydrocarbons in susceptible strains resemble human adenocarcinomas or bronchiolalveolar carcinomas. The spectrum of mutations observed in these chemically induced tumors is similar to human lung carcinomas, including activating point mutations in the *K-ras* oncogene, deletion or altered methylation of the $p16^{INK4a}$ tumor suppressor gene, and deletions at the *FHTT* tumor suppressor gene locus (Tuveson & Jacks, 1999), in addition to increased global LOH, reflecting chromosomal instability (Herzog et al., 2002). Transgenic mouse models have been generated that show an increased incidence of spontaneous lung tumors with a reduced latency. These transgenic mouse models include targeted expression of SV40 T antigen, activated *H-ras* oncogene, mutant *p53* tumor suppressor gene (Tuveson & Jacks, 1999), and deleted $p16^{INK4a}$ tumor suppressor gene (Sharpless et al., 2001). The reduced latency for lung tumor development in genetically susceptible (Malkinson, 2001) and genetically engineered mice (Tuveson & Jacks, 1999) raises the possibility that these mouse strains would be useful for short-term in vivo screening assays for potentially carcinogenic fibers.

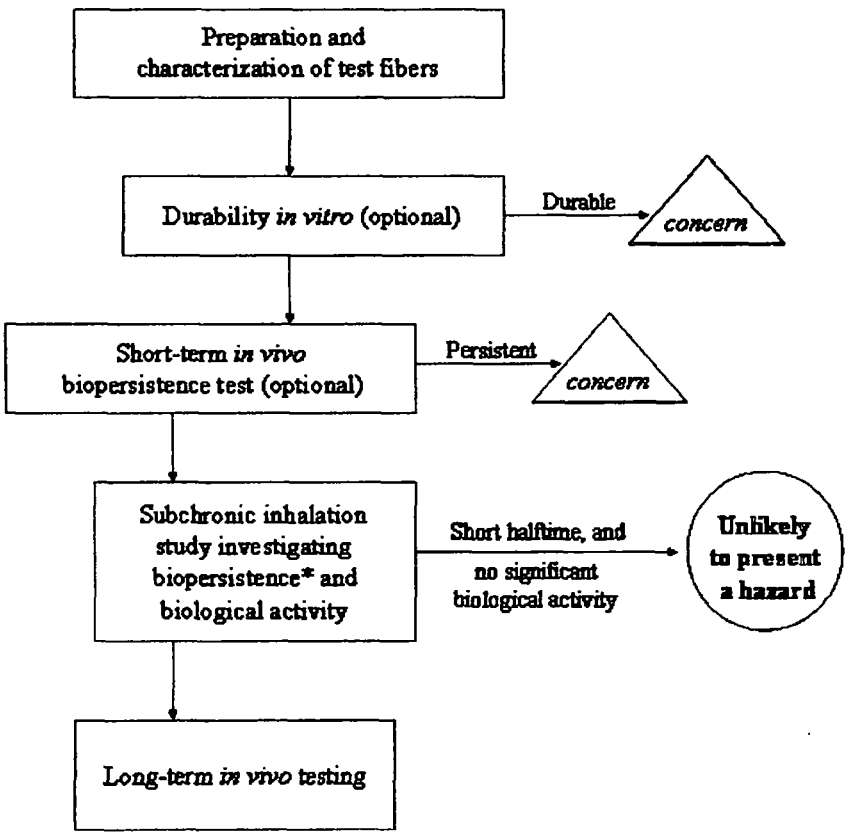
A limited number of transgenic mouse models have recently been evaluated as alternative models for carcinogenicity testing (reviewed in Cohen et al., 2001; Pritchard et al., 2003). Of these transgenic models, the heterozygous $p53^{+/-}$ mouse and the transgenic rasH2 mouse may be useful for assessing the carcinogenicity of fibers. Disruption of the *p53* gene interferes with cell cycle checkpoints, DNA repair, and apoptosis induced by genotoxic agents. The $p53^{+/-}$ heterozygous knockout mouse shows tissue and carcinogen specificity for tumor induction, with a latency of 26 wk in response to chemical carcinogens. LOH is frequently observed at the disrupted *p53* gene locus on chromosome 11, in addition to other mutations in oncogenes and tumor suppressor genes (reviewed in French et al., 2001). The $p53^{+/-}$ heterozygous mouse shows increased sensitivity and accelerated progression of mesotheliomas induced by direct intraperitoneal injection of asbestos fibers (Vaslet et al., 2002). Ras is a key GTP-binding protein in the MAPK signaling pathway; ras is activated by binding of growth factors to tyrosine kinase receptors, leading to cell proliferation. Activating point mutations in the *ras* oncogene cause constitutive activation of the MAPK pathway. The rasH2 transgenic mouse shows elevated, constitutive expression of ras in all tissues and an elevated tumor incidence in response to genotoxic and nongenotoxic carcinogens. The human *H-ras* transgene frequently develops point mutations in chemically induced tumors. The rasH2 transgenic mouse is highly susceptible to lung tumors induced by urethane (Mori et al., 2000). Although neither of these transgenic mice

has yet been exposed to pulmonary carcinogens by intratracheal instillation or inhalation, short-term screening assays may be feasible in these genetically-engineered strains. Molecular assays for LOH at the *p53* tumor suppressor gene locus or genetic alterations in additional oncogenes or tumor suppressor genes may be used as sensitive surrogate markers for carcinogenicity after short-term exposure to synthetic fibers. These molecular markers could be readily assessed over the same time frame as fiber biopersistence, provided that these assays are first validated with appropriate positive and negative controls (e.g., crocidolite asbestos and wollastonite fibers).

7. TESTING STRATEGY

Having described the wide range of short-term testing methods that have been applied to fibers, we now suggest a straightforward strategy for integration of recommended methods in a regulatory hazard evaluation and prioritization scheme for fibers. The testing strategy proposed here reflects the state of the science outlined in the earlier portions of the document. The concepts described are based primarily on the availability of a robust database associated with experimental pulmonary toxicity studies in rats conducted with asbestos fibers and silica-based synthetic vitreous fibers (also known as SVFs; see Figure 1). Through the knowledge gained from studies on these two classes of fibers, some fundamental "testing concepts" have been developed that are likely to be applicable to other fiber-types. In this regard, the available experimental database of fiber toxicity studies strongly suggests that two parameters are particularly important, namely, biopersistence of inhaled fibers in the lung, and the extent and persistence of pulmonary inflammation. Additional factors that play an important role in the development of fiber-related lung disease include proliferation of bronchiolar epithelial cells and interstitial fibrosis. Thus, testing for these parameters should be an integral component of any short-term assay hazard evaluation strategy. Moreover, the specific strategy that will be required for a given fiber type will be dictated by what is known about that fiber type and, in addition, will be influenced by information on similar fiber types.

Two schematic representations of the basic testing strategies that are required to assess the potential toxicity of a given SVF (Figure 6) or "other" (non-SVF) fiber types (Figure 7) are presented in Figures 6 and 7. The preferred species is the rat.



* Unless already studied (in the short-term *in vivo* biopersistence test).

FIG. 6. Tiered testing system for silica-based synthetic vitreous fibers (SVFs).

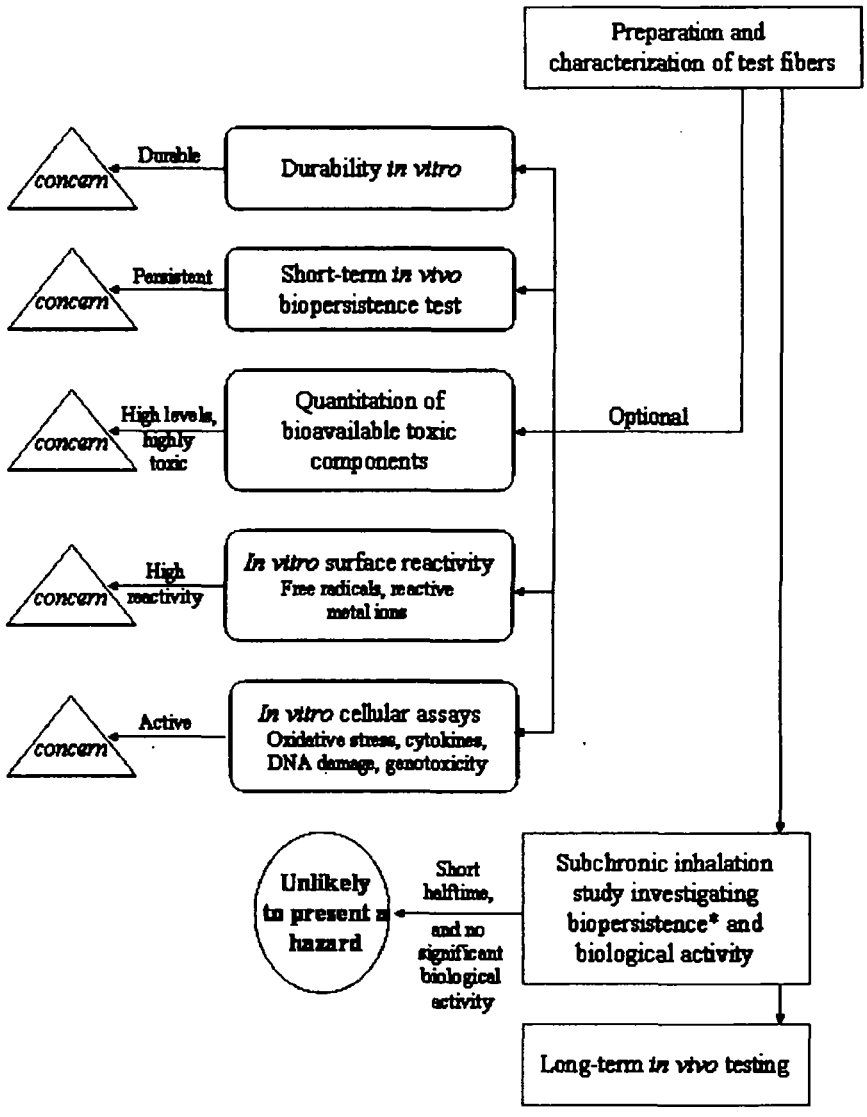


FIG. 7. Tiered testing system for other (non-SVF) fibers.

For all fiber types, the first step of the evaluation process is the preparation and characterization (physical and chemical) of the test fibers. The test fibers should be well-characterized, rat-respirable fibers of appropriate dimensions (e.g., a significant amount >20 μm in length for SVFs), but with relevance to the airborne fibers to which humans are exposed. Subsequently, an in vitro durability test and other in vitro cell-free and cellular assessments are optional, although they may be useful for predicting the toxic potential of a given fiber type. However, it should be noted that, with the exception of the in vitro durability tests for SVFs, most of these in vitro tests do not have standardized protocols or outcomes and have not been validated, or necessarily correlated with in vivo-based results. Still, such tests may assist in prioritizing fibers for further testing, particularly if a battery of tests is performed on a large number of newly developed fiber types.

A short-term in vivo biopersistence test is also optional, and can be obviated by consolidating the in vivo biopersistence assessment with a subchronic inhalation toxicity study (1–3 mo exposure duration, with a postexposure recovery evaluation) that focuses on pulmonary biological endpoints and effects. These two tests form the fundamental components and decision tree of the proposed testing strategy. If the fiber type has a short clearance half-time in the lung and demonstrates no significant

biological activity, then the results would suggest that this fiber type is unlikely to present a significant health hazard. Alternatively, if the results of the study indicate that the fiber type is biopersistent in the lung or presents significant sustained biological activity, such as inflammation, cell proliferation, or pulmonary fibrosis, then there is a cause for concern, and implementation of a chronic inhalation test is likely to be necessary for regulatory evaluation.

For the non-SVF, "other" fiber-types, a suggested schematic is presented in Figure 7. The testing strategy herein is essentially the same as described for SVF fiber-types (Figure 6), with the exception that the optional studies [i.e., (1) durability in vitro; (2) short-term in vivo biopersistence test; (3) quantitation of bioavailable toxic components; (4) in vitro free radical generation and surface reactivity; and (5) in vitro cellular assays] may take on added importance in providing insights into the biological activity of the fiber type in vivo. Nonetheless, as with the testing strategy for SVF fiber types, the subchronic inhalation study, investigating both biopersistence and biological activity of the fiber type in the lung, represents the major criterion for hazard evaluation. Similar to the strategy proposed for the SVF fiber type, the results of the subchronic inhalation study will determine whether additional long-term testing is required.

Given the strategies just outlined, in the following sections we highlight the major parameters that can be assessed in the short-term assays. Parameters are grouped as (1) physicochemical characterization, (2) in vitro toxicity tests, and (3) short-term in vivo animal studies. Those that are considered essential for hazard assessment are identified as "key parameters." Other parameters that may be useful in studying mechanisms of toxicity or in new product development as screening tools for some types of fibers are also noted. These parameters are considered optional in the testing strategies for hazard assessment (Figures 6 and 7). Comments on the utility and applicability of the various assay systems are included for each group of parameters.

7.1. Physicochemical Characterization

The importance and applicability of data on the physical and chemical characteristics of a fiber type in relation to its potential toxicity and carcinogenicity are discussed in sections 5 and 6.3. The importance of fiber geometry, especially length, along with the ability to persist in the milieu of the lung, a function of composition, are the two key parameters currently seen to best predict how harmful a fiber is likely to be. The key parameters identified next enable a degree of quantification of these determinants. Not all of these parameters may be relevant for a given test fiber; for example, cleavage does not apply to amorphous fibers, and if there are no transition metals, then chelation studies are not warranted.

Fiber Morphology

Key Parameters

- *Size distribution of fibers characteristic of human exposure.* The concentration and size distribution of the fibers to which humans are exposed in the work environment should be determined through routine industrial hygiene monitoring if the fiber is already in production. If the fiber is in development, the concentration in the pilot plant should be determined. Anticipated applications of the material should be summarized, including any processes that may affect fiber physicochemical properties (cutting, drilling, sawing, heating, etc.). If technically feasible, simulations of the intended use of the fiber should be conducted to assess the potential human exposure.
- *Bivariate length/diameter distribution.* A complete characterization of the bivariate distribution of fiber lengths and diameters and of the fraction of nonfibrous particulate in the bulk product and the aerosol measurements should be determined. The preferred counting and sizing methods are those outlined in the EC Inhalation Biopersistence Protocol (http://ecb.jrc.it/DOCUMENTS/Testing-Methods/mmmfweb.pdf).
- *Crystallinity, fracture habit.* The structure of the fiber should be specified, in particular, whether the fiber is amorphous or crystalline. In addition, the cleavage and fracture habit of the fiber should be determined in order to assess the potential for creating respirable fibers from larger fibers. (Cleavage and fracture differ in that cleavage is the break of a crystal face where a new face [resulting in a smooth plane] is formed, whereas fracture is the "chipping" of the fiber.)

Chemical Properties

Key Parameters

- *Chemical composition.* The chemical composition of the fibers should be determined very precisely (>99.5%), particularly in the presence of elements that may speciate as toxic moieties in vivo (e.g. arsenic, chromium, etc.). Known or anticipated variation in the chemical composition should be stated.
- *Durability of SVFs in Simulated Body Fluids.* In general, these models have not been considered robust enough to be used for regulatory classification of fibers. However, they have been useful as a first step in internal screening by the fiber industry in developing new SVF fiber compositions.
  - *Calculation of dissolution rate based on composition.* Models exist to calculate fiber dissolution rate directly from composition for a wide range of glass wool and stone wool compositions.
  - *Measurement of dissolution rate in a cell-free system.* The most widely used methods are based on chemical analysis of the dissolution fluid for one or more of the dissolving fiber components in a dynamic flow-through system, but flow-through

methods based on measurement of mass loss or fiber diameter reduction have also been developed and used. Interlaboratory comparisons have shown consistency within laboratories but high interlaboratory variation. Several studies have shown a good correlation between the results of in vitro, cell-free dissolution measurements and fiber durability in the lung (Sebastian et al., 2002; Guldberg et al., 2003).

Other Relevant Properties. Physicochemical parameters that may be useful in further characterizing some types of fibers are the extension of the exposed surface and surface activity.

- *Surface area.* The specific surface can be determined by adsorption of nitrogen at low temperature (BET method) or, as a rough estimate, from electron microscopy information on fiber lengths and diameters. It is useful—when it is the surface of the fiber that determines the biological response—to provide the evaluation of the doses/exposures in terms of exposed surface, for comparing the effects of different fiber types.
- *Surface activity.* Surface activity measurements most relevant to the potential for fiber toxicity are:
  - *Free radical generation.* The release of free radicals from the fibers in aqueous suspension is measured in order to determine the part of oxidative stress caused directly by the fibers without the intervention of macrophages. Sustained and high free radical release (ROS mainly) will damage surrounding cells (lipids, proteins, DNA).
  - *Ion mobilization.* Selective mobilization of transition metal ions or other moieties from the surface by chelators indicates that endogenous molecules may chelate and translocate surface ions to target cells.

Other potentially useful parameters are the extent of surface-reactive metal ions, the surface charge in a physiological solution, and the degree of hydrophilicity/hydrophobicity, which governs protein adsorption and denaturation at the fiber surface.

The parameters just described may be helpful for further characterization, particularly in the case of crystalline and biodurable, noncrystalline inorganic fibers or surface-treated inorganic fibers.

For organic fibers, in the absence of metal impurities, hydrophobicity and surface charge are important; no data are available so far on the generation of free radicals.

Comment. There is currently no single physicochemical parameter that can be used to predict toxicity and carcinogenicity for all fiber classes. For silica-based vitreous fibers, fiber dimensions and biodurability have been shown to be correlated with persistent inflammation, lung fibrosis, lung cancer, and mesothelioma. Chemical composition is an important determinant of biodurability for this class of fibers. For durable natural inorganic fibers, including asbestos and erionite, mobilization of reactive transition metals, the ability to participate in redox reactions, and hydrophilicity are additional physicochemical properties that have been associated with biologic reactivity and cellular toxicity. For some natural inorganic fibers such as erionite, the physicochemical basis for fiber potency in induction of mesotheliomas is not completely understood. For nonvitreous synthetic fibers, such as silicon carbide, there is even less understanding about the physicochemical basis for fiber cellular toxicity and potent carcinogenicity in experimental animals. The important physicochemical parameters for organic fibers are not understood. These fibers may contain compounds that could affect the immune system. If these or other parameters are proven to be important, then their measurement should form part of the physicochemical testing strategy.

7.2. In Vitro Toxicity Tests

Many in vitro toxicity studies have been developed; however, due to the limitations outlined next, current in vitro test systems have limited usefulness for hazard identification or characterization of dose-response. For some fibers, these tests may help to identify and evaluate possible mechanisms involved in fiber pathogenesis. Mechanistic pathways can be elucidated using in vitro test systems and then validated in animal models. Such mechanistic information should be viewed as a complement to in vivo data for risk assessment and not part of recommended routine testing requirements.

Several endpoints that monitor the in vitro toxicity and reactivity of fibers have been described in section 6.5. In vitro studies can be performed using a number of target cells from unexposed or fiber-exposed animals. Cells of interest include:

1. Pulmonary macrophages—These lung cells are the first line of defense against inhaled particles. Phagocytosis of particles is a critical step in pulmonary clearance. Long fibers, too large to be engulfed, result in frustrated phagocytosis, chronic release of mediators, and failed clearance. Interaction of fibers with pulmonary macrophages results in the generation of oxidants, inflammatory cytokines, and growth factors, which are believed to play critical roles in fiber-induced disease. For these reasons, pulmonary macrophages are a common target cell for in vitro studies.
2. Pleural macrophages—In the case of pleural disease, the pleural macrophage is believed to be critical in phagocytosis and clearance of fibers, which enter the intrapleural space. As with pulmonary macrophages, these phagocytes are believed to be an important source of fiber-induced oxidants, inflammatory cytokines, and growth factors.
3. Bronchial epithelial cells—Fibers preferentially deposit at the bifurcations of the terminal and respiratory bronchioles. Therefore, bronchial epithelial cells are a target. Fiber-induced proliferation and mediator production by exposed bronchial epithelial cells are believed to play a role in fiber pathogenesis.

4. Alveolar epithelial cells—Alveolar type II epithelial cell have been shown to produce oxidants and cytokines in response to fiber exposure. Thus, a role in fiber-induced lung disease has been proposed.
5. Mesothelial cells—Proliferation of mesothelial cells is a hallmark of mesothelioma. Therefore, the effects of fibers on cell growth regulation in mesothelial cells is of interest.
6. Fibroblasts—This alveolar interstitial cell is the source of collagen associated with fiber-induced interstitial fibrosis. Regulation of fibroblast proliferation and secretion of extracellular matrix components is a critical step in fibrotic disease.
7. Cocultures—It is becoming clear that mediators from one type of lung cell can affect the activity and proliferation of another type of lung cell. Such cross-talk plays an important role in the initiation and progression of fiber-induced disease. Therefore, evaluation of the effects of fibers on epithelial cell/macrophage cocultures or fibroblast/macrophage cocultures is of interest.

Endpoints that are commonly evaluated in studies in vitro with fibers include:

1. Cell death—Both apoptosis and necrosis are of interest.
2. Metabolism—Effects of fibers on the generation of oxidants as well as the effect of organic chemicals absorbed on fibers on induction of cytochrome P-450 metabolic pathways have been studied.
3. Phagocytosis—The effects of fiber length on frustrated phagocytosis make up a subject of great interest, since this is believed to impact fiber clearance and toxicity.
4. Proliferation—Disregulation of cell growth is believed to play a role in fibrosis (fibroblasts), lung cancer (airway epithelial cells), and mesothelioma (mesothelial cells).
5. ROS/RNS—Reactive oxygen and nitrogen species have been implicated in DNA damage and induction of inflammatory cytokines and growth factors.
6. Chromosomal damage—Fiber-induced oxidants have been linked to chromosomal damage. In addition, fibers have been shown to interfere with mitotic spindles.
7. Signal transduction pathways—Such pathways regulate the production of message for inflammatory cytokines, growth factors, and regulators of the cell cycle. These transcription signals are sensitive to oxidants, which may be generated by fibers produced during the phagocytosis of fibers.
8. Chemotaxis—Localized influx of inflammatory cells may occur at fiber deposition sites and result in localized oxidant/inflammatory stress.
9. Inflammatory mediator gene expression—Activation of promoter genes for cytokines, such as TNF-α, has been reported in response to fiber exposure in vitro.
10. Genotoxicity—Fiber-induced DNA damage in combination with disregulation of the cell cycle could result in proliferation of mutated cells.

Comment. There are several issues that limit the usefulness of in vitro tests for toxicity screening of fibers. For example, short-term in vitro assays of biological activity cannot allow for differences in biopersistence of fibers, and as a result, some nonbiopersistent fibers that are not pathogenic in vivo are positive in short-term in vitro tests (Hesterberg et al., 1983; Ye et al., 1999). In vitro cellular assays, in fact, have several technical limitations:

1. High doses of fibers are used to obtain a positive response; it is difficult to extrapolate from these high-dose, short-term exposures to low-dose, chronic exposures in vivo.
2. Fiber dose in cellular assays is often expressed in terms of mass of fibers rather than numbers of fibers, creating a major problem in relating in vitro to in vivo dose. In fact, number of long (>20 μm) fibers is a better dose metric to use in comparing potency between fiber types. Number of long fibers per cell is the optimal expression of dose, but number of long fibers per unit surface area of the culture dish is an acceptable alternative.
3. In vitro endpoints (e.g., release of inflammatory mediators, activation of transcription factors, induction of cell proliferation or apoptosis) are measured after a few hours or days, while in vivo responses to biopersistent fibers are sustained over several weeks or months. These endpoints have not been validated as screening assays that are predictive of long-term pathological effects in vivo.
4. The target cells used in short-term cellular assays are difficult to standardize and maintain. Stable primary cultures of mesothelial cells, alveolar cells, or terminal bronchial epithelial cells are not widely available. Cell lines used in the published in vitro studies have been derived from human lung tumors (e.g., A549 cells), spontaneously immortalized cells, or cells transfected with viral oncoproteins (e.g., Met5A) that may alter their genotoxic, apoptotic, or proliferative responses.

With the recent introduction of cDNA microarray assays, gene expression profiles of mesotheliomas in rodents and humans have been generated. This technique has not yet been applied to lung tumors induced by asbestos or other fibers. Genomics and proteomics may eventually lead to identification of more specific biomarkers for fiber-induced lung cancer and malignant mesothelioma. Currently, no specific molecular biomarkers have yet been identified. Although quantitative polymerase chain reaction (PCR) assays for gene amplification and expression have been recently developed, these assays are difficult to apply to heterogeneous cell populations. Isolation of specific preneoplastic and early neoplastic cell populations from the lung requires highly specialized techniques (e.g., laser capture microdissection) that will be difficult to adapt for routine screening assays.

In conclusion, current in vitro test systems have limited usefulness for hazard identification or determination of dose

response. However, they may be useful tools to identify and evaluate possible mechanisms involved in fiber pathogenesis. Mechanistic pathways can be elucidated using in vitro test systems and then validated in animal models. Such mechanistic information should be viewed as a scientific complement to in vivo data for risk assessment.

7.3a. Short-Term In Vivo Animal Studies: Fiber Endpoints

As reflected in the proposed testing strategies (Figures 6 and 7), the fiber sample to be tested should be well characterized physically and chemically (section 7.1) with appropriate fiber dimensions for the in vivo studies (e.g., rodent-respirable with a significant fraction longer than 20 μm for SVFs, but also relevant to human exposures). When special preparation of fiber samples is necessary to obtain test materials that are rodent respirable, it is important to be certain that the chemical composition and other inherent characteristics of the fibers have not changed.

Key Parameter

- *Biopersistence.* Measuring the biopersistence of a fiber in the lung is important in characterizing the retained dose. Biopersistence measurements have to be standardized, as the results are highly dependent on the type of application (inhalation or intratracheal instillation), the diameter and length distribution of the test sample, the observation period (timing of scheduled sacrifices), the statistics used for the evaluation of the half-time (see Comment below), and the fiber length selected as the basis for the calculation (e.g., fibers >5 μm or >20 μm). In the European Union, special guidelines have been developed to determine biopersistence after inhalation and intratracheal instillation in rats, which clearly specify the fiber preparation, exposure, and testing parameters in order to provide a highly standardized reproducible protocol for regulatory use. Biopersistence can be measured in a separate short-term study (e.g., 5-day inhalation exposure), or in the subchronic inhalation toxicity study by including subgroups for assessment of lung fiber burden after a 5-day exposure, at exposure termination (1–3 mo), and at the end of the postexposure recovery period.

Other Parameter

- *Pleural fiber burden.* Principally, the number of retained fibers in the pleura can be determined by special techniques. However, the number is expected to be very low after a 5-day inhalation exposure and might be not very meaningful. (After 180 days, the number that have migrated to the pleura may be sufficient to be a useful measure.)

Comment. Short-term in vivo assays to measure the retained dose of fibers in the lung after intratracheal instillation or inhalation have been developed and validated for silica-based vitreous fibers. For this class of fibers, fiber length is a major determinant of carcinogenicity. Experimental data are limited for other classes of synthetic or organic fibers. However, in consideration of additional factors that can contribute to persistent inflammation and fibrosis, the role of shorter fibers and particulates should be evaluated more thoroughly.

A major data gap in the current short-term in vivo assays using natural or synthetic inorganic fibers is the paucity of information on fiber burden and biologic responses in the pleural space and mesothelial lining. The biologic and toxicologic responses of different macrophage populations (alveolar vs. pleural, resident vs. elicited) must be investigated.

Clearance of poorly soluble particles and fibers is much slower from the alveolar region of the respiratory tract than from the ciliated tracheobronchial region. After a short-term exposure in rats, a significant fraction of the fibers is typically deposited in the tracheobronchial region, and the majority of these fibers are rapidly removed by ciliary clearance. This fraction of fibers does contribute to the fast phase of a biphasic lung clearance. For hazard assessment, the slow phase is most relevant and should be evaluated rather than using a weighted half-time of the fast and the slow clearance. However, if only a small fraction (<5%) of the total fiber burden is in the slower clearance component, the weighted half-time may be appropriate.

7.3b. Short-Term In Vivo Animal Studies: Toxicological Endpoints

For short-term in vivo testing of fibers for toxicological endpoints the inhalation route is preferred, since it is the most physiologically based. However, for test fibers available only in low numbers intratracheal instillation is an acceptable alternative mode of delivery, provided that low doses similar to those achievable by inhalation are used and provided that appropriate methods are employed to control and assess possible agglomeration of fibers which can result in artifacts. In either case, the exposure should deliver sufficient long fibers to avoid a false negative. In the case of inhalation exposure this requires a minimum exposure duration of 1 mo and preferably 3 mo. The European Commission 90-day inhalation protocol (European Commission, 1999b) recommends 150 fibers/cm^3 with length greater than 20 μm as the highest of 3 exposure levels in studies of synthetic mineral fibers. A recovery period of at least 3 mo should also be included as part of the study to evaluate the persistence or recovery of key parameters. The key parameters have been validated in the 90-day study for SVFs and asbestos, but the validity for other classes of fibers is less defined and requires further research. In general, further work to validate and standardize this most important subchronic inhalation toxicology study is recommended.

Key Parameters. At various time points throughout the exposure period and postexposure, the following endpoints should be evaluated to assess potential toxic effects in the lungs. These endpoints are in common use, and their interpretation is

generally based on a significant difference from control lungs. During the recovery period the key parameters should return toward baseline for nonpersistent, nontoxic fibers.

- *Lung weight.* This parameter provides a direct, if insensitive, measure of treatment-related change that reflects edema/inflammation or fibrosis.
- *Bronchoalveolar lavage (BAL) profile.* This method samples the cells and fluid from the bronchoalveolar space and allows the assessment of inflammation by quantification of cell numbers and types and components of the fluid phase. In addition, considerable extra information can be gained by various ex vivo manipulations of the BAL cells (e.g., gene expression, phagocytic potential, etc.).
- *Proliferation.* Increased cell division plays a key role in pathological response and can be determined in epithelial or mesothelial cells by uptake of labeled nucleotide precursors, such as tritiated thymidine or BrdU.
- *Fibrosis.* Fibrosis can be determined in lung tissue by measurement of hydroxyproline in lung extracts, by specific staining of collagen in histopathological slides, or by qualitative and quantitative histopathology. However, the hydroxyproline technique is not accurate in detecting small amounts of fibrosis, as is likely to be the case here, so this may be of limited value. Confocal microscopy has been shown to be a sensitive technique for evaluation of fibrosis.
- *Histopathology.* Description of the general effects of treatments on the lungs should include endpoints such as presence of dust-laden macrophages, cellular infiltrates, and hyperplastic change in the epithelium. The Wagner scale represents a tried-and-tested method for quantifying such change.

Other. Other assays identified as having the potential to provide additional information in short-term exposure studies include: 8-hydroxy-deoxyguanosine; HPRT mutation; BAL leukocyte function ex vivo; pleural lavage profile; pleural leukocyte function ex vivo; and clearance of tagged particles.

Comment. The most commonly used endpoints in short-term in vivo assays are inflammation, cell proliferation, and fibrosis; however, newer endpoints may provide additional, useful information.

Inflammatory and immunological reactions in the lungs and pleura may potentially amplify the toxicity of inhaled fibers, leading to fibrosis. Persistent inflammatory responses may also contribute to oxidant stress and secondary genetic and epigenetic changes associated with carcinogenicity. These parameters have been evaluated primarily in the rat. The potential for organic fibers to elicit immunological responses has not been evaluated. Whether asbestosis contributes to the development of lung cancer in humans is controversial, although lung fibrosis has been closely correlated with lung tumors in chronic rat inhalation assays. Adaptive responses (induction of antioxidant defenses and lung cell proliferation) have been documented in short-term inhalation assays in rats exposed to asbestos fibers (Quinlan et al., 1995; Bellmann et al., 2003). These adaptive responses may alter the rate of development of lung tumors or mesotheliomas.

Because the persistent nature of the lesions and the fiber burden of the most hazardous fibers are critical to the evaluation of the potential toxicity of a fiber type, the study design should include a recovery group of animals. This is typically accomplished by including as part of the core group of exposed animals, a group that is held unexposed for at least 3 mo, and possibly up to 12 mo, postexposure. The key parameters evaluated in the recovery animals will be typically dictated by the findings at the end of the exposure, although most of those just listed are usually required. Comparison of postrecovery lung fiber burden with that at exposure termination is also recommended. Specific time points for evaluation during the recovery period will vary with the type of fiber being studied.

This recovery period is necessary to determine whether effects seen at the highest concentration of a three-dose subchronic study will persist, progress, or regress. For example, even a biosoluble fiber may show significant effects at the end of a 3-mo exposure, including some fibrosis, which will regress in a subsequent recovery period. In contrast, similar effects induced by a biopersistent fiber will progress or persist.

REFERENCES

Abbas, A. K., Murphy, K. M., and Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787–793.

Adamson, I. Y., Bakowska, J., and Bowden, D. H. 1993. Mesothelial cell proliferation after instillation of long or short asbestos fibers into mouse lung. *Am. J. Pathol.* 142:1209–1216.

Aldieri, E., Ghigo, D., Tomatis, M., Prandi, L., Fenoglio, I., Costamagna, C., Pescarmona, G., Bosia, A., and Fubini, B. 2001. Iron inhibits the nitric oxide synthesis elicited by asbestos in N11 macrophages. *Free Radical Biol. Med.* 31:412–407.

Ault, J. G., Cole, R. W., Jensen, C. G., Jensen, L. C., Bachert, L. A., and Rieder, C. L. 1995. Behavior of crocidolite asbestos during mitosis in living vertebrate lung epithelial cells. *Cancer Res.* 55:792–798.

Aust A. E., and Eveleigh, J. F. 1999. Mechanism of DNA oxidation. *Exp. Biol. Med.* 222:246–252.

Bellmann, B., and Muhle, H. 1994. Investigation of the biodurability of wollastonite and xonotlite. *Environ. Health Perspect.* 102:191–195.

Bellmann, B., Creutzenberg, O., Dasenbrock, C., Ernst, H., Pohlmann, G., and Muhle, H. 2000. Inhalation tolerance study for p-aramid respirable fiber-shaped particulates (RFP) in rats. *Toxicol. Sci.* 54:237–250.

Bellmann, B., Muhle, H., Creutzenberg, O., Ernst, H., Brown, R. C., and Sebastien, P. 2001. Effects of nonfibrous particles on ceramic fiber (RCF1) toxicity in rats. *Inhal. Toxicol.* 13:877–901.

Bellmann, B., Muhle, H., Ernst, H., Pohlmann, G., Sebastian, P., and Brown, R. C. 2002. Subchronic studies on man-made vitreous fibers: Kinetics of inhaled particles. *Ann. Occup. Hyg.* 46(suppl. 1):166–169.

Bellmann, B., Muhle, H., Creutzenberg, O., Ernst, H., Müller, M., Bernstein, D. M., and Riego Sintes, J. M. 2003. Calibration study on

subchronic inhalation toxicity of man-made vitreous fibers in rats. *Inhal. Toxicol.* 15:1147–1177.

Bernstein, D. M., Morscheidt, C., Grimm, H. G., and Teichert, U. 1996. The evaluation of soluble fibres using the inhalation biopersistence model, a nine fibre comparison. *Inhal. Toxicol.* 8:345–385.

Bernstein, D. M., Morscheidt, C., De Meringo, A., Schumm, M., Grimm, H. G., Teichert, U., Thevenaz, P., and Mellon, L. 1997. The biopersistence of fibers following inhalation and intratracheal instillation exposure. *Ann. Occup. Hyg.* 41:224–230.

Bernstein, D. M., Riego-Sintes, J. M., Ersboell, B. K., and Kunert, J. 2001a. Biopersistence of synthetic mineral fibers as a predictor of chronic inhalation toxicity in rats. *Inhal. Toxicol.* 13:823–849.

Bernstein, D. M., Riego-Sintes, J. M., Ersboell, B. K., and Kunert, J. 2001b. Biopersistence of synthetic mineral fibers as a predictor of chronic intraperitoneal injection tumour response in rats. *Inhal. Toxicol.* 13:851–875.

Bernstein, D. M., Thevenaz, P., Fleissner, H., Anderson, R., Hesterberg, T. W., and Mast, R. 1995. Evaluation of the oncogenic potential of man-made vitreous fibres: The inhalation model. *Ann. Occup. Hyg.* 39:661–672.

BéruBé, K. A., Quinlan, T. R., Moulton, G., Hemenway, D., O'Shaughnessy, P., Vacek, P., and Mossman, B. T. 1996. Comparative proliferative and histopathologic changes in rat lung after inhalation of chrysotile and crocidolite asbestos. *Toxicol. Appl. Pharmacol.* 137:67–74.

Boutin, C., Dumortier, P., Rey, F., Viallat, J. R., and DeVuyst, P. 1996. Black spots concentrate oncogenic asbestos fibers in the parietal pleura: Thoracoscopic and mineralogic study. *Am. J. Respir. Crit. Care Med.* 153:444–449.

Boylan, A. M., Ruegg, C., Kim, K. J., Hebert, C. A., Hoeffel, J. F., Pytela, R., Sheppard, P., Goldstein, I. M., and Broaddus, V. C. 1992. Evidence of a role for mesothelial cell-derived interleukin-8 in the pathogenesis of asbestos-induced pleurisy in rabbits. *J. Clin. Invest.* 89:1257–1267.

Boylan, A. M., Sanan, D. A., Sheppard, D., and Broaddus, V. C. 1995. Vitronectin enhances internalization of crocidolite asbestos by rabbit pleural mesothelialcells via the integrin $\alpha v \beta 5$. *J. Clin. Invest.* 96:1987–2001.

Broaddus, V. C., Yang, L., Scavo, L. M., Ernst, J. D., and Boylan, A. M. 1996. Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species. *J. Clin. Invest.* 98:2050–2059.

Brochard, P., and Bignon, J. 1995. Proposal of a tiered approach to assessing and classifying the health risk of exposure to fibres. *Ann. Occup. Hyg.* 39:737–745.

Brody, A. R., Overby, L. H., and Warheit, D. B. 1987. Inhaled asbestos attracts macrophages and induces rapid proliferation of bronchiolar-alveolar cells. *Chest* 91:302–302.

Brody, A. R., Liu, J.-Y., Brass, D., and Corti, M. 1997. Analyzing the genes and peptide growth factors in lung cells *in vivo* consequent to asbestos exposure and *in vitro*. *Environ. Health Perspect.* 105 (suppl. 5):1165–1171.

Brown, D. M., Roberts, N. K., and Donaldson, K. 1998a. Effect of coating with lung lining fluid on the ability of fibres to produce a respiratory burst in rat alveolar macrophages. *Toxicol. In Vitro* 12:15–24.

Brown, D. M., Fisher, C., and Donaldson, K. 1998b. Free radical activity of synthetic vitreous fibers: Iron chelation inhibits hydroxyl radical generation by refractory ceramic fiber. *J. Toxicol. Environ. Health* 53:545–561.

Brown, D. M., Beswick, P. H., and Donaldson, K. 1999. Induction of nuclear translocation of NF-kappaB in epithelial cells by respirable mineral fibres. *J. Pathol.* 189:258–264.

Brown, D. M., Beswick, P. H., Bell, K. S., and Donaldson, K. 2000. Depletion of glutathione and ascorbate in lung lining fluid by respirable fibres. *Ann. Occup. Hyg.* 44:101–108.

Brown, G. M., Cowie, H., Davis, J. M., and Donaldson, K. 1986. In vitro assays for detecting carcinogenic mineral fibres: A comparison of two assays and the role of fibre size. *Carcinogenesis* 7:1971–1974.

Brown, R. C., Sara, E. A., Hoskins, J. A., Evans, C. E., Young, J., Laskowski, J. J., Acheson, R., Forder, S. D., and Rood, A. P. 1992. The effects of heating and devitrification on the structure and biological activity of aluminosilicate refractory ceramic fibres. *Ann. Occup. Hyg.* 36(2):115–129.

Castellan, R. M., Olenchock, S. A., Hankinson, J. L., Millner, P. D., Cooke, J. B., Bragg, K. C., Perkins, H. H., and Jacobs, R. R. 1984. Acute bronchoconstriction induced by cotton dust: Dose-related responses to endotoxin and other dust factors. *Ann. Intern. Med.* 101:157–163.

Castellan, R. M., Olenchock, S. A., Kingsley, K. B., and Hankinson, J. L. 1987. Inhaled endotoxin and decreased spirometric values. An exposure-response relationship for cotton dust. *N. Engl. J. Med.* 317:605–610.

Chao, C. C., and Aust, A. E. 1994. Effect of long term removal of iron from asbestos by desferrioxamine B on subsequent mobilization by other chelators and induction of DNA single strand breaks. *Arch. Biochem. Biophys.* 308:64–69.

Chao, C. C., Park, S. H., and Aust, A. E. 1996. Participation of nitric oxide and iron in the oxidation of DNA in asbestos-treated human lung cells. *Arch. Biochem. Biophys.* 326:152–157.

Cheng, N., Shi, X., Ye, J., Castranova, V., Chen, F., Leonard, S. S., Vallyathan, V., and Rojanasakul, Y. 1999. Role of transcription factor NF-kappaB in asbestos-induced TNFalpha response from macrophages. *Exp. Mol. Pathol.* 66:201–210.

Churg, A., and Green, F. H. Y. 1998. *Pathology of occupational lung disease*, 2nd ed., pp. 235–402. Baltimore MD: Williams & Wilkins.

Churg, A. M., and Warnock, M. L. 1981. Asbestos and other ferruginous bodies: Their formation and clinical significance. *Am. J. Pathol.* 102:447.

Cohen, S. M., Robinson, D., and MacDonald, J. 2001. Alternative models for carcinogenicity testing. *Toxicol. Sci.* 64:14–19.

Coin, P. G., Roggli, V. L., and Brody, A. R. 1994. Persistence of long, thin chrysotile asbestos fibers in the lungs of rats [Review]. *Environ. Health Perspect.* 102(suppl. 5):197–199.

Costa, D., Guignard, J., Zalma, R., and Pezerat, H. 1989. Production of free radicals arising from the surface activity of minerals and oxygen. Part I. Iron mine ores. *Toxicol. Ind. Health* 5(6):1061–1078.

Council of the European Union. 1997. Commission Directive 97/69/EC. *Off. J. Eur. Commun.* L343/19–L343/20. 13-12-0097.

Coussens, L. M., and Werb, Z. 2003. Inflammation and cancer. *Nature* 420:860–867.

Crapo, J. D., Young, S. L., Fram, E. K., Pinkerton, K. E., Barry, B. E., and Crapo, R. O. 1983. Morphometric characteristics of cells in the alveolar region of mammalian lungs. *Am. Rev. Respir. Dis.* 128:S42–S46.

Creutzenberg, O., Bellmann, B., and Muhle, H. 1998. Biopersistence and bronchoalveolar lavage invetigations in rats after a subacute inhalation of various man-made mineral fibres. *Ann. Occup. Hyg.* 41(suppl. 1):213–218.

Dai, Y. T., and Yu, C. P. 1998. Alveolar deposition of fibers in rodents and humans. *J. Aerosol. Med.* 11(4):247–258.

Davila, R. M., and Crouch, E. C. 1993. Role of mesothelial and submesothelial stromal cells in matrix remodeling following pleural injury. *Am. J. Pathol.* 142:547–555.

Davis, J. M., Addison, J., Bolton, R. E., Donaldson, K., Jones, A. D., and Smith, T. 1986. The pathogenicity of long versus short fibre samples of amosite asbestos administered to rats by inhalation and intraperitoneal injection. *Brit. J. Exp. Pathol.* 67:415–430.

Davis, J. M. G. 1996. Mixed fibrous and non-fibrous dust exposures and interactions between agents in fibre carcinogensis. In *Mechanisms of fibre carcinogenesis*, eds. A. B. Kane, P. Boffetta, R. Saracci, and J. D. Wilburn, pp. 127–135. Lyon: IARC Scientific Publication 140.

Davis, J. M. G., and Cowie, H. A. 1990. The relationship between fibrosis and cancer in experimental animals exposed to asbestos and other fibers. *Environ. Health Perspect.* 88:305–309.

Davis, J. M. G., Brown, D. M., Cullen, R. T., Donaldson, K., Jones, A. D., Miller, B. G., McIntosh, C., and Searl, A. 1996. A comparison of methods for determining and predicting the pathogenicity of mineral fibres. *Inhal. Toxicol.* 8:747–770.

de Meringo, A., Morscheidt, C., Thélohan, S., and Tiesler, H. 1994. In vitro assessment of biodurability: Acellular systems. *Environ. Health Perspect* 102 (Suppl. 5):47–53.

Ding, M., Dong, Z., Chen, F., Pack, D., Ma, W.Y., Je, J., Shi, X., Castranova, V., and Vallyathan, V. 1999a. Asbestos induces activator protein-transactivation in transgenic mice. *Cancer Res.* 59:1884–1889.

Ding, M., Shi, X., Dong, Z., Chen, F., Lu, Y., Castranova, V., and Vallyathan, V. 1996b. Freshly fractured crystalline silica induces activator protein activation through erks and p38 MAPK. *J. Biol. Chem.* 274:30611–30616.

Donaldson, K., and Brown, G. M. 1993. Bronchoalveolar lavage in the assessment of the cellular response to fiber exposure. In: *Fiber toxicology*, ed. D. B. Warheit, pp. 117–138. New York: Academic Press.

Donaldson, K., Li, X. Y., Dogra, S., Miller, B. G., and Brown, G. M. 1992. Asbestos-stimulated tumor-necrosis-factor release from alveolar macrophages depends on fiber length and opsonization. *J. Pathol.* 168:243–248.

Donaldson, K., Beswick, P. H., and Gilmour, P. S. 1996. Free radical activity associated with the surface of particles: A unifying factor in determining biological activity? *Toxicol. Lett.* 88:293–298.

Dopp, E., Saedler, J., Stopper, H., Weiss, D. G., and Schiffmann, D. 1995. Mitotic disturbances and micronucleus induction in Syrian hamster embryo fibroblast cells caused by asbestos fibers. *Environ. Health Perspect.* 103:268–271.

Driscoll, K. E., Deyo, L. C., Carter, J. M., Howard, B. W., Hassenbein, D. G., and Bertram, T. A. 1997. Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. *Carcinogenesis* 18:423–430.

Driscoll, K. E., Costa, D. L., Hatch, G., Henderson, R., Oberdorster, G., Salem, H., and Schlesinger, R. B. 2000. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: Uses and limitations. *Toxicol. Sci.* 55:24–35.

Driscoll, K. E., Carter, J. M., and Borm, P. J. A. 2002. Antioxidant defense mechanisms and the toxicity of fibrous and nonfibrous particles. *Inhalation Toxicol.* 14:101–118.

Eastes, W., Potter, R. M., and Hadley, J. G. 2000a. Estimating rock and slag wool fiber dissolution rate from composition. *Inhal. Toxicol.* 12:1127–1139.

Eastes, W., Potter, R. M., and Hadley, J. G. 2000b. Estimation of dissolution rate from in vivo studies of synthetic vitreous fibers. *Inhal. Toxicol.* 12:1037–1054.

Eastes, W., Potter, R. M., and Hadley, J. G. 2000c. Estimating in vitro glass fiber dissolution rate from composition. *Inhal. Toxicol.* 12:269–280.

Eborn, S. K., and Aust, A. E. 1995. Effect of iron acquisition on induction of DNA single strand breaks by erionite, a carcinogenic mineral fiber. *Arch. Biochem. Biophys.* 316:507–514.

Elias, Z., Poirot, O., Daniere, M. C., Terzetti, F., Binet, S., Tomatis, M., and Fubini, B. 2002. Surface reactivity, cytotoxicity, and transforming potency of iron-covered compared to untreated refractory ceramic fibers. *J. Toxicol. Environ. Health A* 65(23):2007–2027.

Eschenbacher, W. L., Kreiss, K., Lougheed, M. D., Pransky, G. S., Day, B., and Castellan, R. M. 1999. Nylon flock-associated interstitial lung disease. *Am. J. Respir. Crit. Care Med.* 159:2003–2008.

European Commission. 1997. Commission Directive 97/69/EC of 5.XII.97 (23rd adaptation) O.J. L 343/1997.

European Commission. 1999a. Biopersistence of fibres: Short-term exposure by inhalation (ECB/TM/26 rev. 7). In *Methods for the determination of the hazardous properties for human health of man made mineral fibers (MMMF)*, European Commission Joint Research Centre, report EUR 18748 EN (1999), eds. D. M. Bernstein and J. M. Riego Sintes. http://ecb.jrc.it/testing-methods

European Commission. 1999b. Sub-chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats (ECB/TM/16(97) rev. 1). In *Methods for the determination of the hazardous properties for human health of man made mineral fibers (MMMF)*. European Commission Joint Research Centre, report EUR 18748 EN (1999), eds. D. M. Bernstein and J. M. Riego Sintes. http://ecb.jrc.it/testing-methods

Fenoglio, I., Prandi, L., Tomatis, M., and Fubini, B. 2001. Free radical generation in the toxicity of inhaled mineral particles: The role of iron speciation at the surface of asbestos and silica. *Redox Rep.* 6(4):235–241.

Fischer, J. J., Foarde, K., Ellakkani, M., Ogundiran, N., and Karol, M. H. 1986. Comparison of artificial cotton dusts for causing acute respiratory reactions. In *Proceedings of the 10th Cotton Dust Research Conference*, January 8–9, 1986, Las Vegas, NV, eds. R. R. Jacobs and P. J. Wakelyn, pp. 119–121. Memphis, TN: National Cotton Council.

Fisher, C. E., Brown, D. M., Shaw, J., Beswick, P. H., and Donaldson, K. 1998. Respirable fibres: Surfactant coated fibres release more Fe^{3+} than native fibres at both pH 4.5 and 7.2. *Ann. Occup. Hyg.* 42:337–345.

Fisher, C. E., Rossi, A. G., Shaw, J., Beswick, P. H., and Donaldson, K. 2000. Release of TNFalpha in response to SiC fibres: Differential effects in rodent and human primary macrophages, and in macrophage-like cell lines. *Toxicol. In Vitro.* 14:25–31.

French, J., Storer, R. D., and Donehower, L. A. 2001. The nature of the heterozygous "Trp" 53 knockout model for identification of mutagenic carcinogens. *Toxicol. Pathol.*. 29(suppl.):24–29.

Fubini, B. 1996. Use of physico-chemical and cell free assays to evaluate the potential carcinogenicity of fibres. In *Mechanisms of fibre*

carcinogenesis, eds. A. B. Kane, P. Boffetta, R. Saracci, and J. D. Wilbourn, pp. 35–54. Lyon: IARC Scientific Publication 140.

Fubini, B., and Mollo, L. 1995. Role of iron in the reactivity of mineral fibers. *Toxicol. Lett.* 82–83:951–960.

Fubini, B., and Otero-Aréan, C. 1999. Chemical aspects of the toxicity of inhaled mineral dusts. *Chem. Soc. Rev.* 28:373–381.

Fubini, B., Mollo, L., and Giamello, E. 1995. Free radical generation at the solid/liquid interface in iron containing minerals. *Free Radical Res.* 23:593–614.

Fubini, B., Barcelò, F., and Otero-Arean, C. 1997. Ferritin adsorption on amosite fibers: Possible implications in the formation and toxicity of asbestos bodies *J. Toxicol. Environ. Health* 52:101–110.

Fubini, B., Aust, A. E., Bolton, R. E., Borm, P. J. A., Bruch, J., Ciapetti, G., Donaldson, K., Elias, Z., Gold, J., Jaurand, M. C., Kane, A. B., Lison, D., and Muhle, H. 1998. Non-animal tests for evaluating the toxicity of solid xenobiotics. ECVAM Workshop Report 30. *Altern. Lab. Anim.* 26:579–617.

Fubini, B., Zanetti, G., Altilia, S., Tiozzo, R., Lison, D., and Saffiotti, U. 1999. Relationship between surface properties and cellular responses to crystalline silica: Studies on heat-treated cristobalite. *Chem. Res. Toxicol.* 12:737–745.

Fung, H., Kow, Y. W., Van Houten, B., and Mossman, B. T. 1997. Patterns of 8-hydroxydeoxyguanosine formation in DNA and indications of oxidative stress in rat and human pleural mesothelial cells after exposure to crocidolite asbestos. *Carcinogenesis* 18:825–832.

Garn, H., Friedetzky, A., Davis, G. S., Hemenway, D. R., and Gemsa, D. 1997. T-lymphocyte activation in the enlarged thoracic lymph nodes of rats with silicosis. *Am. J. Respir. Cell Mol. Biol.* 16:309–316.

Gazdar, A. F., Butel, J. S., and Carbone, M. 2002. SV40 and human tumours: Myth, association or causality? *Nat. Rev. Cancer* 2:957–964.

Gelzleichter, T. R., Bermudez, E., Mangum, J. B., Wong, B. A., Everitt, J. I., and Mass, O. R. 1996. Pulmonary and pleural responses in Fischer 344 rats following short-term inhalation of a synthetic vitreous fiber. I. Quantitation of lung and pleural fiber burdens. *Fundam. Appl. Toxicol.* 30:31–38.

Gelzleichter, T. R., Bermudez, E., Mangum, J. B., Wong, B. A., Janszen, D. B., Moss, O. R., and Everitt, J. I. 1999. Comparison of pulmonary and pleural responses of rats and hamsters to inhaled refractory ceramic fibers. *Toxicol. Sci.* 49:93–101.

Ghio, A. J., LeFurgey, A., and Roggli, V. L. 1997. In vivo accumulation of iron on crocidolite is associated with decrements in oxidant generation by the fiber. *J. Toxicol. Environ. Health* 50:125–142.

Ghio, A. J., Kadiiska, M. B., Xiang, Q.-H., and Mason, R.P. 1998. In vivo evidence of free radical formation after asbestos instillation: An ESR spin trapping investigation. *Free Radical Biol. Med.* 24:11–17.

Gilmour, P. S., Beswick, P. H., Brown, D. M., and Donaldson, K. 1995. Detection of surface free radical activity of respirable industrial fibres using supercoiled phi X174 RF1 plasmid DNA. *Carcinogenesis* 16(12):2973–2979.

Gilmour, P. S., Brown, D. M., Beswick, P. H., MacNee, W., Rahman, I., and Donaldson, K. 1997. Free radical activity of industrial fibers: Role of iron in oxidative stress and activation of transcription factors. *Environ. Health Perspect.* 105:1313–1317.

Gjomarkaj, M., Pace, E., Melis, M., Spatafora, M., Profita, M., Vignola, A. M., Bonsignore, G., and Toews, G. B. 1999. Phenotypic and functional characterization of normal rat ppleural macrophages in comparison with autologous peritoneal and alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 20:135–142.

Goldberg, J. L., Zanella, C. L., Janssen, Y. M. W., Timblin, C. R., Jimenez, L. A., Vacek, P., Taatjes, D. J., and Mossman, B. T. 1997. Novel cell imaging techniques show induction of apoptosis and proliferation in mesothelial cells by asbestos. *Am. J. Respir. Cell Mol. Biol.* 17:265–271.

Greim, H, Borm, P., Schins, R., Donaldson, K., Driscoll, K., Hartwig, A., Kuempel, E., Oberdorster, G., and Speit, G. 2001. Toxicity of fibers and particles. Report of the workshop held in Munich, Germany, 26–27 October 2000. *Inhal. Toxicol.* 13:737–754.

Guldberg, M., Madsen, A. L., Sebastian, K., Fellmann, J., Potter, R., Bauer, J., Searl, A., Maquin, B., and Jubb, G. 2003. In-vitro dissolution of vitreous silicate fibres according to EURIMA test guideline—Results of two round robins. *Glass Sci. Technol.* 76:199–205.

Gulumian, M. 1999. The ability of mineral dusts and fibres to initiate lipid peroxidation. Part I: Parameters which determine this ability. *Redox Rep.* 4(4):141–163.

Gulumian, M., Bhoolia, D. J., Du Toit, R. S. J., Rendall, R. E. G., Pollak, H., van Wyk, J. A., and Rhempula, M. 1993a. Activation of UICC crocidolite: The effect of conversion of some ferric ions to ferrous ions. *Environ. Res* 60:193–206.

Gulumian, M., van Wyk, J. A., Hearne, G. R., Kolk, B., and Pollak, H. 1993b. ESR and Mossbauer studies on detoxified crocidolite: Mechanism of reduced toxicity. *J. Inorg. Biochem.* 50:133–143.

Halliwell, B., and Gutteridge, J. M. 1986. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Arch. Biochem. Biophys.* 246(2):501–514.

Hancock, A., Armstrong, L., Gama, R., and Millar, A. 1998. Production of interleukin 13 by alveolar macrophages from normal and fibrotic lung. *Am. J. Respir. Cell Mol. Biol.* 18:60–65.

Hansen, K., and Mossman, B. T. 1987. Generation of superoxide (O_2^-.) from alveolar macrophages exposed to asbestiform and nonfibrous particles. *Cancer Res* 47:1681–1686.

Hardy, J. A., and Aust, A. E. 1995a. Iron in asbestos chemistry and carcinogenicity. *Chem. Rev.* 95:97–118.

Hardy, J. A., and Aust, A. E. 1995b. The effect of iron binding on the ability of crocidolite asbestos to catalyse DNA single-strand breaks. *Carcinogenesis* 16(2):319–325.

Hartwig, A. 2002. Role of DNA repair in particle-and fiber-induced lung injury. *Inhal. Toxicol.* 14:91–100.

Hei, T. K., He, Z. Y., and Suzuki, K. 1995. Effects of antioxidants on fiber mutagenesis. *Carcinogenesis* 16:1573–1578.

Hei, T. K., Xu, A., Louie, D., and Zhao, Y.-L. 2000. Genotoxicity versus carcinogenicity; implications from fiber toxicity studies. *Inhal. Toxicol.* 12(suppl. 3):141–147.

Heintz, N. H., Janssen, Y. M., and Mossman, B. T. 1993. Persistent induction of c-fos and c-jun expression by asbestos. *Proc. Natl. Acad. Sci USA* 90:3299–3303.

Herzog, C. R., Devereux, T. R., Pittman, B., and You, M. 2002. Carcinogenic induction directs the selection of allelic losses in mouse lung tumorigenesis. *Cancer Res.* 62:6424–6429.

Hesterberg, T. W., and Barrett, J.C. 1984. Dependence of asbestos- and mineral dust-induced transformation of mammalian cells in culture on fiber dimension. *Cancer Res.* 44:2170–2180.

Hesterberg, T. W., Miller, W. C., McConnell, E. E., Chevalier, J., Hadley, J. G., Bernstein, D. M., Thevenaz, P., and Anderson, R. 1993. Chronic

inhalation toxicity of size-separated glass fibers in Fischer 344 rats. *Fundam. Appl. Toxicol.* 20:464–476.

Hesterberg, T. W., Oshimura, M., and Barrett, J. C. 1985. Asbestos induces anaphase abnormalities in mammalian cells in culture at doses which induce cell-transformation and heteroploidy. *Environ. Mutagen.* 7:88–89.

Hesterberg, T. W., Miiller, W. C., Musselman, R. P., Kamstrup, O., Hamilton, R. D., and Thevenaz, P. 1996. Biopersistence of man-made vitreous fibers and crocidolite asbestos in the rat lung following inhalation. *Fundam. Appl. Toxicol.* 29:267–279.

Hesterberg, T. W., Chase, G., Axten, C., Miller, W. C., Musselman, R. P., Kamstrup, O., Hadley, J., Morscheidt, C., Bernstein, D. M., and Thevenaz, P. 1998a. Biopersistence of synthetic vitreous fibers and amosite asbestos in the rat lung following inhalation. *Toxicol. Appl. Pharmacol.* 151:262–275.

Hesterberg, T. W., Hart, G. A., Chevalier, J., Miiller, W. C., Hamilton, R. D., Bauer, J., and Thevenaz, P. 1998b. The importance of fiber biopersistence and lung dose in determining the chronic inhalation effects of X607, RCF1, and chrysotile asbestos in rats. *Toxicol. Appl. Pharmacol.* 153:68–82.

Hesterberg, T. W., Hart, G. A., Miiller, W. C., Chase, G., Rogers, R. A., Mangum, J. B., and Everitt, J. I. 2002. Use of short-term assays to evaluate the potential toxicity of two new biosoluble glasswool fibers. *Inhal. Toxicol.* 14:217–246.

Hill, I. M., Beswick, P. H., and Donaldson, K. 1995. Differential release of superoxide anions by macrophages treated with long and short fibre amosite asbestos is a consequence of differential affinity for opsonin. *Occup. Environ. Med.* 52(2):92–96.

Holian, A., Uthman, M. O., Goltsova, T., Brown, S. D., and Hamilton, R. F., Jr. 1997. Asbestos and silica-induced changes in human alveolar macrophage phenotype. *Environ. Health Perspect.* 105(suppl. 5):1139–1142.

Hu, Y. C., Sidransky, D., and Ahrendt, S. A. 2002. Molecular detection approaches for smoking associated tumors. *Oncogene* 21:7289–7297.

Huaux, F., Lardot, C., Arras, M., Delos, M., Many, M. C., Coutelier, J. P., Buchet, J. P., Renauld, J. C., and Lison, D. 1999. Lung fibrosis induced by silica particles in NMRI mice is associated with an upregulation of the p40 subunit of interleukin-12 and Th-2 manifestations. *Am. J. Respir. Cell Mol. Biol.* 20:561–572.

Hubbard, A. K., Timblin, C. R., Shukla, A., Rincon, M., and Mossman, B. T. 2002. Activation of NF-kappaB-dependent gene expression by silica in lungs of luciferase reporter mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 282:L968–L975.

IARC. 1987. Asbestos. *IARC Monogr. Eval. Carcinogen. Risks Hum.* (Suppl. 7):106–116.

IARC. 1997. Silica, some silicates, coal dust and para-aramid fibrils. *IARC Monogr. Eval. Carcinogen. Risks Hum.* 68:441.

IARC. 1999. Surgical implants and other foreign bodies. *IARC Monogr. Eval. Carcinogen. Risks Hum.* 74:313–317.

IARC. 2002. Man-made vitreous fibres. *IARC Monogr. Eval. Carcinogen. Risks Hum.* 81:43–80, 339.

Ilgren, E., and Chatfield, E. 1998. Coalinga fibre—A short, amphibole-free chrysotile. Part 2. Evidence for lack of tumourigenic activity. *Indoor Built Environ.* 7:18–31.

Jacobs, R. R., Boehlecke, B., Van Huge-Hamsten, M., and Rylander, R. 1993. Bronchial reactivity, atropy, and airway response to cotton dust. *Am. Rev. Respir. Dis.* 148:19–24.

Jaurand, M. C. 1994. In-vitro assessment of biopersistence using mammalian-cell systems. *Environ. Health Perspect.* 102:55–59.

Jaurand, M. C. 1996. Use of in-vitro genotoxicity and cell transformation assays to evaluate the potential carcinogenicity of fibers. In *Mechanisms of fibre carcinogenesis, IARC Scientific Publications No. 140*, eds. A. B. Kane, P. Boffetta, R. Saracci, and J. D. Wilburn, pp. 55–72. Lyon, France: IARC Sci. Pub. 140.

Jensen, C. G., Jensen, L. C. W., Reider, C. L., Cole, R. W., and Ault, J. G. 1996. Long crocidolite fibers cause polyploidy by sterically blocking cytokinesis. *Carcinogenesis* 17:2013–2021.

Johnson, N. F., and Hahn, F. F. 1996. Induction of mesothelioma after intrapleural inoculation of F344 rats with silicon carbides whiskers or continuous ceramic filaments. *Occup. Environ. Med* 53:813–816.

Kamp, D. W., and Weitzman, S. A. 1999. The molecular basis of asbestos induced lung injury. *Thorax* 54:638–652.

Kamstrup, O., Davis J. M. G., Ellehauge, A., and Guldberg, M. 1998. The biopersistence and pathogenicity of man-made vitreous fibres after short- and long-term inhalation. *Ann. Occup. Hyg.* 42:191–199.

Kandaswami, C., and O'Brien, P. J. 1983. Effect of chrysotile asbestos and silica on the microsomal metabolism of benzo(a)pyrene. *Environ. Health Perspect.* 51:311–314.

Kane, A. B. 1996. Mechanisms of mineral fibre carcinogenesis. In: *Mechanisms of fibre carcinogenesis*, eds. A. B. Kane, P. Boffetta, R. Saracci, and J. D. Wilbourn, pp. 11–34. France: IARC Scientific Publication 140.

Kane, A. B. 2000. Oncogenes and tumor suppressor genes in the carcinogenicity of fibers and particles. *Inhal. Toxicol.* 12(suppl. 3):133–140.

Karin, M., Yixue, C., Greten, F. R., and Li, Z.-W. 2002. NF-κB in cancer: From innocent bystander to major culprit. *Nat. Rev. Cancer* 2:301–310.

Keeling, B., Li, K. Y., and Churg, A. 1994. Iron enhances uptake of mineral particles and increases lipid peroxidation in tracheal epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 10:683–688.

Kern, D. G., Kuhn, C., III, Ely, E. W., Pransky, G. S., Mello, C. J., Fraire, A. E., and Muller, J. 2000. Flock worker's lung: Broadening the spectrum of clinicopathology, narrowing the spectrum of suspected etiologics. *Chest* 117:251–259.

Kjuus, H., Skjaerven, R., Langard, S., Lien, J. T., and Aamodt, T. 1986. A case-referent study of lung cancer, occupational exposures and smoking. II. Role of asbestos exposure. *Scand. J. Work Environ. Health* 12:203–209.

Kodama, Y., Boreiko, C. J., Maness, S. C., and Hesterberg, T. W. 1993. Cytotoxic and cytogenetic effects of asbestos on human bronchial epithelial cells in culture. *Carcinogenesis* 14:691–697.

Krombach, F., Münzing, S., Allmeling, A. M., Gerlach, J. T., Behr, J., and Dörger, M. 1997. Cell size of alveolar macrophages: An interspecies comparison. *Environ. Health Perspect* 105(suppl. 5):1261–1263.

Lakowicz, J. R., and Bevan, D. R. 1979. Effects of asbetos, iron oxide, silica, and carbon black on the microsomal availability of benzo[a]pyrene. *Biochemistry* 18:5170–5176.

Lakowicz, J. R., Bevan, D. R., and Riemer, S. C. 1980. Transport of a carcinogen, benzo[a]pyrene, from particulates to lipid bilayers: A model for the fate of particle-adsorbed polynuclear aromatic hydrocarbons which are retained in the lungs. *Biochim. Biophys. Acta* 629:243–258.

Lange, J. H. 1988. A review of epidemiological evidence of anti-cancer properties of dust. In *Proceedings of the 12th Cotton Dust Research Conference, January 6–7, 1988, New Orleans, LA*, eds. R. R. Jacobs and P. J. Wakelyn, pp. 124–127. Memphis, TN: National Cotton Council.

Lanone, S., Zheng, T., Zhu, Z., Liu, W., Lee, C. G., Ma, B., Chen, Q., Homer, R. J., Wang, J., Rabach, L. A., Rabach, M. E., Shipley, J. M., Shapiro, S., Senior, R. M., and Elias, J. A. 2002. Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and-12 in IL-13-induced inflammation and remodeling. *J. Clin. Invest.* 110:463–474.

Lapin, C. A., Craig, D. K., Valerio, M. G., McCandless, J. B., and Bogoroch, R. 1991. A subchronic inhalation toxicity study in rats exposed to silicon carbide whiskers. *Fundam. Appl. Toxicol.* 16:128–146.

Lasky, J. A., Bonner, J. C., Tonthat, B., and Brody, A. R. 1996. Chrysotile asbestos induces pdgf-a chain-dependent proliferation in human and rat lung fibroblasts in-vitro. *Chest* 109:26S–28S.

Leanderson, P., Soderkvist, P., Tagesson, C., and Axelson, O. 1988. Formation of DNA adduct 8-hydroxy-2′-deoxyguanosine induced by man-made mineral fibres. In *Methods for detecting DNA damaging agents in humans: Applications in cancer epidemiology and prevention*, eds. H. Bartsch, K. Hemminki, and I.K. O'Neil, pp. 422–424. Lyon: IARC Press.

Lee, B. W., Wain, J. C., Kelsey, K. T., Weincke, J. K., and Christiani, D. C. 1998. Association of cigarette smoking and asbestos exposure with location and histology of lung cancer. *Am. J. Respir. Crit. Care Med.* 157:748–755.

Lee, C. G., Homer, R. J., Zhu, Z., Lannone, S., Wang, X., Koteliansky, V., Shipley, J. M., Gotwals, P., Nobels, P., Chen, Q., Senior, R. M., and Elias, J. A. 2001. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor β1. *J. Exp. Med.* 194:809–821.

Lee, K. P., Barras, C. E., Griffith, F. D., Waritz, R. S., and Lapin, C. A. 1981. Comparative pulmonary responses to inhaled inorganic fibers with asbestos and fiberglass. *Environ. Res* 24:167–191.

Leikauf, G. D., Fink, S. P., Miller, M. L., Lockey, J. E., and Driscoll, K. E. 1995. Refractory ceramic fibers activate alveolar macrophage eicosanoid and cytokine release. *J. Appl. Physiol* 78:164–171.

Levresse, V., Renier, A., Fleury-fieth, J., Levy, f., Moritz, S., Vivo, C., Pilatte, Y., and Jaurand, M. C. 1997. Analysis of cell cycle disruptions in cultures of rat pleural mesothelial cells exposed to asbestos fibers. *Am. J. Respir. Cell Mol. Biol.* 17:660–671.

Liu, J. Y., Morris, G. F., Lei, W. H., Corit, M., and Brody, A. R. 1996. Up-regulated expression of transforming growth factor-α in the bronchiolar-alveolar duct regions of asbestos-exposed rats. *Am. J. Pathol.* 149:205–217.

Liu, J. Y., Morris, G. F., Lei, W. H., Hart, C. E., Lasky, J. A., and Brody, A. R. 1997. Rapid activation of PDGF-A and -B expression at sites of lung injury in asbestos-exposed rats. *Am. J. Respir. Cell Mol. Biol.* 17:129–140.

Lu, L., Keane, M. J., Ong, T., and Wallace, W. E. 1994. In vitro genotoxicity studies of chrysotile asbestos fibres dispersed in simulated pulmonary surfactant. *Mut. Res.* 320:253–259.

Lum, H., Tyler, W. S., Hyde, D. M., and Plopper, C. G. 1983. Morphometry of *in situ* and lavaged pulmonary alveolar macrophages from control and ozone-exposed rats. *Exp. Lung Res.* 5:61–78.

Lund, L. G., and Aust, A. E. 1992. Iron mobilization from crocidolite asbestos greatly enhances crocidolite-dependent formation of DNA single-strand breaks in phi X174 RFI DNA. *Carcinogenesis* 13:637–642.

Lund, L. G., Williams, M. G., Dodson, R. F., and Aust, A. E. 1994. Iron associated with asbestos bodies is responsible for the formation of single strand breaks in ϕ X174 RFI DNA. *Occup. Environ. Med.* 51:200.

Luoto, K., Holopainen, M., Karppinen, K., Perander, M., and Savolainen, K. 1994. Dissolution of man-made vitreous fibers in rat alveolar macrophage culture and Gamble's saline solution: Influence of different media and chemical composition of the fibers. *Environ. Health Perspect.* 102(suppl. 5):103–107.

Luoto, K., Holopainen, M., and Savolainen, K. 1995. Durability of man-made vitreous fibers as assessed by dissolution of silicon, iron and aluminium in rat alveolar macrophages. *Ann. Occup. Hyg.* 39:855–867.

Luoto, K., Holopainen, M., Kangas, J., Kalliokoski, P., and Savolainen, K. 1998. Dissolution of short and long rockwool and glasswool fibers by macrophages in flowthrough cell culture. *Environ. Res.* A78:25–37.

Luster, M. I., and Simeonova, P. P. 1998. Asbestos induces inflammatory cytokines in the lung through redox sensitive transcription factors. *Toxicol. Lett.* 102–103:271–275.

Malkinson, A. M. 2001. Primary lung tumors in mice as an aid for understanding, preventing, and treating human adenocarcinoma of the lung. *Lung Cancer* 32:265–279.

Manning, C. B., Vallyathan, V., and Mossman, B. T. 2002. Disease caused by asbestos: Mechanisms of injury and disease development. *Int. Immunopharmacol.* 2:191–200.

Maples, K. R., and Johnson, N. F. 1992. Fiber-induced hydroxyl radical formation—Correlation with mesothelioma induction in rats and humans. *Carcinogenesis* 13:2035–2039.

Martra, G, Tomatis, M., Fenoglio, I., Coluccia, S., and Fubini, B. 2003. Ascorbic acid modifies the surface of asbestos: Possible implications in the molecular mechanisms of toxicity. *Chem. Res. Toxicol.* 16:328–335.

Mattson, S. M. 1995. Factors affecting fiber dissolution—In-vitro experiments. In *Proceedings of the XVII International Congress on Glass, Outubro, 1995*, vol. 3, pp. 368–373. Pequim, China: Chinese Ceramic Society.

Maxim, L. D., Mast, R. W., Utell, M. J., Yu, C. P., Boymel, P. M., Zoilos, B. K., and Cason, J. E. 1999. Hazard assessment and risk analysis of two new synthetic vitreous fibers. *Regul. Toxicol. Pharmacol.* 30:54–74.

McClellan, R. O., Miller, F. J., Hesterberg, T. W., Warheit, D. B., Bunn, W. B., Kane, A. B., Lippmann, M., Mast, R. W., Mcconnell, E. E., and Reinhardt, C. F. 1992. Approaches to evaluating the toxicity and carcinogenicity of man-made fibers—Summary of a workshop held November 11–13, 1991, Durham, North Carolina. *Regul. Toxicol. Pharmacol.* 16:321–364.

McConnell, E. E. 1994. Synthetic vitreous fibers—Inhalation studies. *Regul. Toxicol. Pharmacol.* 20:S22–S34.

McConnell, E. E., Wagner, J. C., Skidmore, J. W., and Moore, J. A. 1984. A comparative study of the fibrogenic and carcinogenic effects of UICC Canadian chrysotile B asbestos and glass microfibre (JM 100). In *Biological effects of man-made mineral fibres*, vol. 2, pp. 234–252. Geneva: World Health Organization.

McConnell, E. E., Hall, L., and Adkins, B. 1991. Studies on the chronic toxicity (inhalation) of wollastonite in Fischer 344 rats. *Inhal. Toxicol.* 3:323–337.

McConnell, E. E., Axten, C., Hesterberg, T. W., Chevalier, J., Miiller, W. C., Everitt, J., Oberdorster, G., Chase, G. R., Thevenaz, P., and Kotin, P. 1999. Studies on the inhalation toxicology of two fiberglasses and amosite asbestos in the Syrian golden hamster. Part II. Results of chronic exposure. *Inhal. Toxicol.* 11:785–835.

Meldrum, M. 2002. *Review of fibre toxicology.* Sudbury, UK: HSE Books.

Merchant, J. A., Kilburn, K. H., O'Fallen, W. M., Hamilton, J. D., and Lumsden, J. C. 1972. Byssinosis and chronic bronchitis among cotton textile workers. *Ann. Intern. Med.* 76:423–433.

Miller, B. G., Jones A. D., Searl, A., Buchanan D., Cullen, R. T., Soutar, C. A., Davis J. M. G., and Donaldson, K. 1999. Influence of characteristics of inhaled fibres on development of tumours in the rat lung. *Ann. Occup. Hyg.* 43:167–179.

Morgan, A. 1997. Acid leaching studies of chrysotile asbestos from mines in the Coalinga region of California and from Quebec and British Columbia. *Ann. Occup. Hyg.* 41:249–268.

Mori, I., Yasuhara, K., Hayashi, S. M., Nonoyama, T., Nomura, T., and Mitsumori, K. 2000. Carcinogen dose-dependent variation in the transgene mutation spectrum in urethane-induced lung tumors in transgenic mice carrying the human prototype c-Ha-ras gene. *Cancer Lett.* 153:199–209.

Mosser, D. M. 2003. The many faces of macrophages activation. *J. Leukocyte Biol.* 73:209–212.

Mossman, B. T., Eastman, A., and Bresnick, E. 1984. Asbestos and benzo[a]pyrene act synergistically to induce squamous metaplasia and incorporation of [^{3}H]thymidine in hamster tracheal epithelium. *Carcinogenesis* 5:1401–1404.

Mossman, B. T., Faux, S., Janssen, Y., Jimenez, L. A., Timblin, C., Zanella, C., Goldberg, J. Walsh, E., Barchowsky, A., and Driscoll, K. 1997. Cell signaling pathways elicited by asbestos. *Environ. Health Perspect.* 105(suppl. 5):1121–1125.

Muhle, H., and Bellmann, B. 1995. Biopersistence of man-made vitreous fibers. *Ann. Occup. Hyg.* 39:655–660.

Muhle, H., Pott, F., Bellmann, B., Takenaka, S., and Ziem, U. 1987. Inhalation and injection experiments in rats to test the carcinigenicity of MMMF. *Ann. Occup. Hyg.* 31:755–764.

Muhle, H., Bellmann, B., and Pott, F. 1994. Comparative investigations of the biodurability of mineral fibers in the rat lung. *Environ. Health Perspect.* 102:163–168.

Muhle H., Ernst, H., and Bellmann, B. 1997. Investigation of the durability of cellulose fibres in rat lungs. *Ann. Occup. Hyg.* 41(suppl. 1):184–188.

Nehls, P., Seiler, F., Rehn, B., Greferath, R., and Bruch, J. 1997. Formation and persistence of 8-oxoguanine in rat lung cells as an important determinant for tumor formation following particle exposure. *Environ. Health Perspect.* 105(suppl. 5):1291–1296.

Nejjari, A., Fournier, J., Pezerat, H., and Leanderson, P. 1993. Mineral fibres: Correlation between oxidising surface activity and DNA base hydroxylation. *Br. J. Ind. Med.* 50:501–504.

Nelson, H. H., and Kelsey, K. T. 2002. The molecular epidemiology of asbestos and tobacco in lung cancer. *Oncogene* 21:7284–7288.

NIOSH. 1994. Asbestos and other fibres by PCOM: Method 7400. In *NIOSH manual of analytical methods*, 4th ed., eds. P. M. Eller and M. E. Cassinelli, pp. 94–113. Cincinnati, OH: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, NIOSH.

O'Byrne, K. J., Dalgleish, A. G., Browning, M. J., Steward, W. P., and Harris, A. L. 2000. The relationship between angiogenesis and the immune response in carcinogenesis and the progression of malignant disease. *Eur. J. Cancer* 36:151–169.

Oberdorster, G. 1996. Evaluation and use of animal models to assess mechanisms of fibre carcinogenicity. In *Mechanisms of fibre carcinogenesis*, eds. A. B. Kane, P. Boffetta, R. Saracci, and J. D. Wilbourn, pp. 107–125. Lyon, France: IARC. IARC Scientific Publication 140.

Oberdorster, G. 2000. Determinants of the pathogenicity of man-made vitreous fibers (MMVF). *Int. Arch. Occup. Environ. Health* 73(suppl.):S60–S68.

Oberdörster, G., Ferin, J., Marcello, N. L., and Meinhold, S. H. 1983. Effect of intrabronchially instilled amosite on lavagable lung and pleural cells. *Environ. Health Perspect.* 51:41–48.

Oberdörster, G., Morrow, P. E., and Spurny, K. 1988. Size dependent lymphatic short-term clearance of amosite fibers in the lung. *Ann. Occup. Hyg.* 32 (suppl.):149–156.

Otero-Arean, C., Barcelò, F., and Fubini, B. 1999. Free radical activity of mineral fibres containing adsorbed ferritin: Detection using supercoiled DNA. Res. *Chem. Intermed.* 25:177–185.

Otero-Arean, C., Barcelò, F, Fenoglio, I., Fubini, B., Llabrès, F. X., Xamena, I., and Tomatis, M. 2001. Free radical activity of natural and heat treated amphibole asbestos. *J. Inorg. Biochem.* 83:211–216.

Ottaviani, F., Tomatis, M., and Fubini, B. 2000. Surface properties of vitreous fibres. *J. Colloid Interface Sci.* 224:169–178.

Pache, J. C., Janssen, Y. M. W., Walsh, E. S., Quinlan, T. R., Zanells, C. L., Low, R. B., Taatjes, D. J., and Mossman, B. T. 1998. Increased epidermal growth factor-receptor protein in a human mesothelial cell line in response to long asbestos fibers. *Am. J. Pathol.* 152:333–340.

Palecanda, A., and Kobzik, L. 2000. Alveolar macrophage–environmental particle interaction: Analysis by flow cytometry. *Methods* 21:241–247.

Palecanda, A., Paulauskis, J. Al-Mutairi, E., Imrich, A., Qin, G., Suzuki, H., Kodama, T., Tryggvason, K., Koziel, H., and Kobzik, L. 1999. Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. *J. Exp. Med.* 189:1497–1506.

Park, S. H., and Aust, A. E. 1998. Participation of iron and nitric oxide in the mutagenicity of asbestos in *hgprt*$^-$, *gpt*$^+$ Chinese hamster V79 cells. *Cancer Res.* 58:1144–1148.

Parks, C. G., Conrad, K., and Cooper, G. S. 1999. Occupational exposure to crystalline silica and autoimmune disease. *Environ. Health Perspect.* 107(suppl. 5):793–802.

Pezerat, H., Zalma, R., Guignard, J., and Jaurand, M. C. 1989. Production of oxygen radicals by the reduction of oxygen arising from the surface activity of mineral fibres. *IARC Sci. Publ.* 90:100–111.

Pott, F., Roller, M., Rippe, R. M., Germann, P. G., and Bellmann, B. 1991. Tumours by the intraperitoneal and intrapleural routes and their significance for the classification of mineral fibres. In *Mechanisms in fibre carcinogenesis*, eds. R. C. Brown, J. A. Hoskins and H. F. Johnson, vol. 223, pp. 547–565. New York: Plenum Press.

Potter, R. M. 2000. Method for determination of in-vitro fiber dissolution rate by direct optical measurement of diameter decrease. *Glastech. Ber.* 43:46–55.

Prandi, L., Tomatis, M., Penazzi, N., and Fubini, B. 2002. Iron cycling mechanisms and related modifications at the asbestos surface. *Ann. Occup. Hyg.* 46:140–143.

Pritchard, J. B., French, J. E., Davis, B. J., and Haseman, J. K. 2003. The role of transgenic mouse models in carcinogen identification. *Environ. Health Perspect* 111:444–454.

Puhakka, A., Ollikainen, T., Soini, Y., Kahlos, K., Saily, M., Koistinen, P., Paakko, P., Linnainmaa, K., and Kinnula, V. L. 2002. Modulation of DNA single-strand breaks by intracellular glutathione in human lung cells exposed to asbestos fibers. *Mutat. Res.* 514:7–17.

Quinlan, T. R., Berube, K. A., Marsh, J. P., Janssen, Y. M. W., Taishi, P., and Leslie, K. O. 1995. Patterns of inflammation, cell proliferation, and related gene expression in lung after inhalation of chrysotile asbestos. *Am. J. Pathol.* 147:728–739.

Ramos-Nino, M. E., Timblin, C. R., and Mossman, B. T. 2002. Mesothelial cell transformation requires increased AP-1 binding activity and ERK-dependent Fra-1 expression. *Cancer Res.* 62:6065–6069.

Reddy, S. P. M., and Mossman, B. T. 2002. Role and regulation of activator protein-1 in toxicant-induced responses of the lung. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283:L1161–L1178.

Riganti, C., Aldieri, E., Bergandi, L., Fenoglio, I., Costamagna, C., Fubini, B., Bosia, A., and Ghigo D. 2002. Crocidolite asbestos inhibits pentose phosphate oxidative pathway and glucose 6-phosphate dehydrogenase activity in human lung epithelial cells. *Free Radical Biol. Med.* 32:938–949.

Rihn, B., Coulais, C., Kauffer, E., Bottin, M.-C., Martin, P., Yvon, F., Vigheron, J. C., Binet, S., Monhoven, N., Steiblen, G., and Keith, G. 2000. Inhaled crocidolite mutagenicity in lung DNA. *Environ. Health Perspect.* 108:341–346.

Robledo, R. F., Buder-Hoffmann, S. A., Cummins, A. B., Walsh, E. S., Taatjes, D. J., and Mossman, B. T. 2000. Increased phosphorylated extracellular signal-regulated kinase immunoreactivity associated with proliferative and morphologic lung alterations after chrysotile asbestos inhalation in mice. *Am. J. Pathol.* 156:1307–1316.

Romundstad, P., Andersen, A., and Haldorsen T. 2001. Cancer incidence among workers in the Norwegian silicon carbide industry. *Am. J. Epidemiol.* 153:978–986.

Rylander, R., Schilling, R. S. F., Pickering, C. A. C., Rooke, G. B., Dempsey, A. M., and Jacobs, R. R. 1987. Effects after acute and chronic exposure to cotton dust: The Manchester criteria. *Br. J. Ind. Med.* 12:577–579.

Samet, J. M. 2000. Does idiopathic pulmonary fibrosis increase lung cancer risk? *Am. J. Respir. Crit. Care Med.* 161:1–2.

Sandu, H., Dehnen, W., Roller, M., Abel, J., and Unfried, K. 2000. mRNA expression patterns in different stages of asbestos-induced carcinogenesis in rats. *Carcinogenesis* 21:1023–1029.

Schapira, R. M., Ghio, A. J., Effros, R. M., Morrisey, J., Dawson, C. A., and Hacker, A. D. 1994. Hydroxyl radicals are formed in rat lung after asbestos instillation *in vivo*. *Am. J. Respir. Cell Mol. Biol.* 10:573–579.

Scheule, R. K., and Holian, A. 1990. Modification of asbestos bioactivity for the alveolar macrophage by selective protein adsorption. *Am. J. Respir. Cell Mol. Biol.* 2:441–448.

Scheule, R. K., and Holian, A. 1989. IgG specifically enhances chrysotile asbestos-stimulated superoxide anion production by the alveolar macrophage. *Am. J. Respir. Cell Mol. Biol.* 1:313–318.

Schins, R. P. F., and Donaldson, K. 2000. Nuclear factor kappa B activation by particles and fibres. *Inhal. Toxicol.* 12(suppl. 3):317–326.

Searl, A., Buchanan, D., Cullen, R. T., Jones, A. D., Miller, B. G., and Soutar, C. A. 1999. Biopersistence and durability of nine mineral fibre types in rat lungs over 12 months. *Ann. Occup. Hyg.* 43:143–153.

Sebastian, K., Fellmann, J., Potter, R., Bauer, J., Searl, A., de Meringo, A., Maquin, B., de Reydellet, A., Jubb, G., Moore, M., Preininger, R., Zoitos, B., Boymel, P., Steanberg, T., Madsen, A. L., Guldberg, M. 2002. EURIMA test guideline: In-vitro acellular dissolution of man-made vitreous silicate fibres. *Glass Sci. Technol.* 75:263–270.

Sebring, R. J., and Lehnert, B. E. 1992. Morphometric comparison of rat alveolar macrophages, pulmonary interstitial macrophages, and blood monocytes. *Exp. Lung Res.* 18:479–496.

Sesko, A., Cabot, M., and Mossman, B. 1990. Hydrolysis of inositol phospholipids precedes cellular proliferation in asbestos-stimulated tracheobronchial epithelial cells2. *Proc. Natl. Acad. Sci. USA* 87:7385–7389.

Sime, P. J., and O'Reilly, K. M. 2001. Fibrosis of the lung and other tissues: New concepts in pathogenesis and treatment. *Clin. Immunol.* 99:308–319.

Simeonova, P. P., Toriumi, W., Kommineni, C., Erkan, M., Munson, A. E., Rom, W. N., and Luster, M. I. 1997. Molecular regulation of IL-6 activation by asbestos in lung epithelial cells—Role of reactive oxygen species. *J. Immunol.* 159:3921–3928.

Stanton, M. F., Layard, M., Tegeris, A., Miller, E., May, M., Morgan, E., and Smith, A. 1981. Relation of particle dimension to carcinogenicity in amphibole asbestoses and other fibrous minerals. *JNCI* 67:965–975.

Stone, K. C., Mercer, R. R., Gehr, P., Stockstill, B., and Crapo, J. D. 1992. Allometric relationships of cell numbers and size in the mammalian lung. *Am. J. Respir. Cell Mol. Biol.* 6:235–243.

Timblin, C. R., Janssen, Y. M., and Mossman, B. T. 1995. Transcriptional activation of the protooncogene c-jun by asbestos and H_2O_2 is directly related to increased proliferation and transformation of tracheal epithelial-cells. *Cancer Res.* 55:2723–2726.

Tlsty, T. D. 2001. Stromal cells can contribute oncogenic signals. *Seminars Cancer Biol.* 11:97–104.

Tomatis, M., Prandi, l., Bodoardo, S., and Fubini, B. 2002a. Loss of surface reactivity upon heating amphibole asbestos. *Langmuir* 18:4345–4350.

Tomatis, M., Fenoglio, I., Elias, Z., Poirot, O., and Fubini, B. 2002b. Effect of thermal treatments of refractory ceramic fibres on the induction of cytotoxicity and cell transformation. *Ann. Occup. Hyg.* 46:176–180.

Touray, J. C., and Baillif, P. 1994. In vitro assessment of the biopersistence of vitreous fibers: State of the art from the physical-chemical point of view. *Environ. Health Perspect.* 102(suppl. 5):25–30.

Travis, W. D., Colby, J. V., Koss, M. N., Rosado-de-Christenson, M. L., Müller, N. L., and King, T. E., Jr. 2002. Non-neoplastic disorders of the lower respiratory tract. In *Atlas of nontumor pathology, First series, Fascicle 2*, pp. 814–846. Washington, DC: American Registry of Pathologists and Armed Forces Institute Pathologists.

Tuveson, D. A., and Jacks, T. 1999. Modeling human lung cancer in mice: Similarities and shortcomings. *Oncogene* 18:5318–5324.

Unfried, K., Schürkes, C., and Abel, J. 2002. District spectrum of mutations induced by crocidolite asbestos: Clue for 8-hydroxydeoxyguanosine-dependent mutagenesis *in vivo*. *Cancer Res.* 62:99–104.

U.S. Environmental Protection Agencies. 2001. *OPPTS 870.8355 Combined chronic toxicity/carcinogenicity testing of respirable fibrous particles*. EPA 712-C-01-352. http://www.epa.gov/opptsfrs/home/guidelin.htm.

U.S. Environmental Protection agencies. 1996. *Chemical information collection and development (testing). Additions to the Master Testing List—Categories*. http://www.epa.gov/opptintr/chemtest/mtladcat.htm.

Vallyathan, V., and Shi, X. 1997. The role of oxygen free radicals in occupational and environmental lung disease. *Environ. Health Perspect.* 105(suppl. 1):165–177.

Vaslet, C. A., Messier, N. J., and Kane, A. B. 2002. Accelerated progression of asbestos-induced mesotheliomas in heterozygous $p53^{+/-}$ mice. *Toxicol. Sci.* 68:331–338.

Vaughan, G., Trently, S. A., and Wilson R. B. 1993. Pulmonary response, in vivo, to silicon carbide whiskers. *Environ Res.* 63:191–201.

Vu, V., Barrett, J. C., Roycroft, J., Schuman, L., Dankovic, D., Baron, P., Martonen, T., Pepelko, W., and Lai, D. 1996. Workshop report. Chronic inhalation toxicity and carcinogenicity testing of respirable fibrous particles. *Regul. Toxicol. Pharmacol.* 24:202–212.

Wagner, J. C., Skidmore, J. W., Hill, R. J., and Griffiths, D. M. 1985. Erionite exposure and mesotheliomas in rats. *Br. J. Cancer* 51:727–730.

Warheit, D. B. 1993. Assessments of pulmonary toxicity following short-term exposures to inhaled fibrous materials. In *Fiber toxicology*, ed. D. B. Warheit, pp. 207–228. New York: Academic Press.

Warheit, D. B. 1995. A review of inhalation toxicology studies with para-aramid fibrils. *Ann. Occup. Hyg.* 39:691–697.

Warheit, D. B., Kellar, K. A., and Hartsky, M. A. 1992. Pulmonary cellular effects in rats following aerosol exposures to ultrafine Kevlar® aramid fibrils: Evidence for biodegradability of inhaled fibrils. *Toxicol. Appl. Pharmacol.* 116:225–239.

Warheit, D. B., Hartsky, M. A., and Webb, T. R. 2000. Biodegradability of inhaled *p*-aramid respirable fibre-shaped particulates: Representative of other synthetic organic fibre-types? *Int. Arch. Occup. Environ. Health* 73(suppl.):S75–S78.

Warheit, D. B., Hart, G. A., Hesterberg, T. W., Collins, J. J., Dyer, W. M., Swaen, G. M., Castranova, V., Soiefer, A. I., and Kennedy, G. L., Jr. 2001a. Potential pulmonary effects of man-made organic fiber (MMOF) dusts. *Crit. Rev. Toxicol.* 31:697–736.

Warheit, D. B., Hartsky, M. A., Reed, K. L., and Webb, T. R. 2001b. Biodegradability of inhaled para-aramid RFP: Mechanistic in vivo and in vitro studies. *Toxicol. Appl. Pharmacol.* 174:78–88.

Warheit, D. B., Webb, T. R. Reed, K. L., Hansen, J. F., and Kennedy, G. L., Jr. 2003. Four-week inhalation toxicity study with Nylon RFP in rats: Rapid lung clearance. *Toxicology* 192:189–210.

Weitzman, S. A., and Graceffa, P. 1984. Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide. *Arch. Biochem. Biophys.* 228:373–376.

World Health Organization. 1985. *Reference method for measuring airborne man-made mineral fibres (MMMF)*. Environmental Health Report no. 4. Copenhagen: World Health Organization, Regional Office for Europe.

Ye, J., Shi, X., Jones, W., Rojanasakul, Y., Cheng, N., Schwegler-Berry, D., Baron, Deye, G. J., Li, C., and Castranova, V. 1999. Critical role of glass fiber length in TNF-alpha production and transcription factor activation in macrophages. *Am. J. Physiol.* 276:L426–L434.

Yucesoy, B., Vallyathan, V., Landsittel, D. P., Simeonova, P., and Luster, M. I. 2002. Cytokine polymorphisms in silicosis and other pneumoconiosis. *Mol. Cell. Biochem.* 234/235:219–224.

Zhu, Z., Man, B., Zheng, T., Homer, R. J., Lee, C. G., Charo, I. F., Noble, P., and Elias, J. A. 2002. IL-13-induced chemokine responses in the lung: Roe of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J. Immunol.* 168:2953–2962.

Zoitos, B. K., de Meringo, A., Rouyer, E., Thlohan, S., Bauer, J., Law, B., Boymel, P. M., Olson, J. R., Christensen, V. R., Guldberg, M., Koenig, A. R., and Perander, M. 1997. In-vitro measurement of fiber dissolution rate relevant to biopersistence at neutral pH: An interlaboratory round robin. *Inhal. Toxicol.* 9:525–540.